



Published in final edited form as:

*Nat Rev Cancer*. 2020 May ; 20(5): 263–273. doi:10.1038/s41568-020-0252-3.

## Zebrafish patient avatars in cancer biology and precision cancer therapy

**Maurizio Fazio**<sup>1,2</sup>, **Julien Ablain**<sup>1,2</sup>, **Yan Chuan**<sup>3</sup>, **David M. Lengenau**<sup>3</sup>, **Leonard I. Zon**<sup>1,2,✉</sup>

<sup>1</sup>Stem Cell Program and Division of Hematology/Oncology, Boston Children's Hospital and Dana-Farber Cancer Institute, Howard Hughes Medical Institute, Harvard Medical School, Boston, MA, USA.

<sup>2</sup>Department of Stem Cell and Regenerative Biology, Harvard Stem Cell Institute, Cambridge, MA, USA.

<sup>3</sup>Molecular Pathology Unit, Cancer Center, Massachusetts General Hospital Research Institute, Charlestown, MA, USA.

### Abstract

In precision oncology, two major strategies are being pursued for predicting clinically relevant tumour behaviours, such as treatment response and emergence of drug resistance: inference based on genomic, transcriptomic, epigenomic and/or proteomic analysis of patient samples, and phenotypic assays in personalized cancer avatars. The latter approach has historically relied on in vivo mouse xenografts and in vitro organoids or 2D cell cultures. Recent progress in rapid combinatorial genetic modelling, the development of a genetically immunocompromised strain for xenotransplantation of human patient samples in adult zebrafish and the first clinical trial using xenotransplantation in zebrafish larvae for phenotypic testing of drug response bring this tiny vertebrate to the forefront of the precision medicine arena. In this Review, we discuss advances in transgenic and transplantation-based zebrafish cancer avatars, and how these models compare with and complement mouse xenografts and human organoids. We also outline the unique opportunities that these different models present for prediction studies and current challenges they face for future clinical deployment.

---

The biomedical community has been developing an ever-growing number of novel anticancer agents. After an era where chemotherapy was the only systemic treatment available, a broad range of new targeted therapies and, more recently immunotherapy agents,

---

✉ zon@enders.harvard.edu.

#### Author contributions

M.F. and J.A. researched data for the article, substantially contributed to discussion of the content, wrote the manuscript, and reviewed and edited the manuscript before submission. Y.C. researched data for the article and wrote the manuscript. D.L. substantially contributed to discussion of the content, and reviewed and edited the manuscript before submission. L.I.Z. substantially contributed to discussion of the content, wrote the manuscript, and reviewed and edited the manuscript before submission.

#### Competing interests

L.I.Z. is a founder and stockholder of Fate Therapeutics Inc., Scholar Rock and Camp4 Therapeutics Inc., and is a scientific adviser for Stemgent. The other authors declare no competing interests.

#### Peer review information

*Nature Reviews Cancer* thanks M. G. Ferreira, Z. Gong, R. Stewart and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

have entered clinical development or have received regulatory approval<sup>1,2</sup>. This expanding list of treatment options offers new hope for patients with cancer in need of systemic therapy, but also poses new challenges for oncologists. The deceptively simple question of which drug to prescribe to a specific patient is increasingly harder to address with certainty. Development of new agents is outpacing the long and complex trials required to identify which dosage, schedule and combinatorial strategy will confer the greatest clinical benefit to which defined patient population.

The field of precision oncology was born from the need to address these issues by creating a new prognostic and therapeutic paradigm focused on optimizing treatment for an individual patient, rather than a group. Precision medicine at large philosophically moves away from predictions based on comparison of group averages and focuses instead on a patient's unique disease features. Specifically, in precision oncology this new paradigm has resulted in two major approaches. The first approach is the inference of disease behaviour based on either single or multiple omic (genomic, transcriptomic, epigenomic and proteomic) analyses of tumour biopsy samples<sup>3,4</sup>, which is relatively rapid and inexpensive as a result of improving technologies. The second approach is the functional testing of tumour behaviours and response to drugs using personalized cancer avatars obtained from in vitro cultures or in vivo xenotransplantation of tumour biopsy samples<sup>5-7</sup>. As a clinical prediction tool, the former approach is limited by our knowledge of the functional consequences of individual genetic mutations in genes outside the common cancer drivers, by the effect of combinations of events and by the complexity of clonal dynamics and tumour evolution that can contribute to drug response<sup>8</sup>. The latter approach overcomes this complexity by simply focusing on the tumour's phenotypic behaviour and emulating the possible treatment settings; however, it is constrained by the biological fidelity of specific models as a proxy for patient response and by the logistical considerations of assay duration, cost and scalability and the challenge of testing multiple conditions<sup>5</sup>. Phenotypic testing of drug response has historically relied on growing patient-derived cancer cells in vitro as 2D cultures<sup>9</sup> or 3D organoids<sup>6</sup>, or in vivo as mouse xenografts<sup>5,10,11</sup>. Recent technological advances in the generation of both transgenic<sup>12</sup> and transplantation-based zebrafish cancer avatars<sup>13,14</sup>, combined with the intrinsic logistic advantages of scale, cost, time and multiplexing of conditions<sup>15</sup>, and the potential for automation<sup>16,17</sup> bring zebrafish into the arena of phenotypic testing of drug response for precision cancer therapy.

In this Review, we highlight the history and evolution of tools that have made possible the rapid generation of combinatorial transgenic cancer models in zebrafish<sup>12,18</sup>, and the development of xenotransplantation of patient samples into larvae and adult immunodeficient zebrafish raised at 37 °C (REFS<sup>13,14</sup>). We discuss how zebrafish cancer avatars compare with human organoids and mouse xenografts for drug testing. Finally, we highlight some of the limitations and challenges for future clinical implementation. For detailed coverage of human cancer organoids and xenografts, we refer the reader to the work of colleagues<sup>5,6,10,11,19</sup>.

## Advances in zebrafish cancer avatars

### Stable transgenic cancer models

Due to the extensive conservation with humans of organ-specific genetic programmes and cancer-associated genes<sup>20</sup>, a closer resemblance to humans than mice in telomere biology<sup>21</sup> and the relative ease of genetic manipulation of embryos by microinjection at the one-cell stage, zebrafish quickly emerged as a promising organism to model cancer in vivo<sup>22</sup>. In 2003, the first genetically engineered cancer model in zebrafish of T cell acute lymphoblastic leukaemia (T-ALL) was reported<sup>23</sup> (FIG. 1). The mouse *Myc* open reading frame was placed under control of the zebrafish *rag2* promoter, which restricts expression to lymphoid cells. When this was injected into wild-type zebrafish embryos, 5% of F0 zebrafish developed T-ALL between 1 month and 5 months of age. Germline transmission of the transgene increased the penetrance to 100% and reduced the latency of tumours. This model provided a remarkable proof of concept for cancer modelling in zebrafish and laid the foundation for new discoveries of mechanisms that drive leukaemogenesis. This study also paved the way for developing other tumour models in zebrafish, including pancreatic neuroendocrine tumours<sup>24</sup> in 2004, rapidly followed by melanoma<sup>25</sup> in 2005, and many others have been established since then, including conditional models that use Cre-mediated recombination, and chemically inducible models<sup>26–28</sup>. However, in most human cancers, particularly solid tumours, oncogenesis is thought to require multiple genetic drivers, including both the activation of an oncogene and the loss of a tumour suppressor<sup>29,30</sup>. Accordingly, zebrafish models based solely on the expression of an oncogene, with exception of RAS-driven models<sup>31</sup>, have generally shown poor penetrance, which has considerably restrained their use. Other limitations include the fact that many models were obtained serendipitously by expression of an oncogene that did not always reflect the early clonal genetic drivers of human tumours<sup>24,32</sup>. The degree of tissue specificity of the promoter driving oncogene expression is also critical for the faithful recapitulation of human tumorigenesis.

#### One-cell stage

The zebrafish embryonic stage where the egg has been fertilized by sperm, but before the first cell division.

The first zebrafish model of melanoma used the most common driver found in human melanoma, *BRAF*<sup>V600E</sup>, placed under the control of the melanocyte-specific zebrafish *mitfa* promoter<sup>25</sup>. As in humans, *BRAF*<sup>V600E</sup> expression in zebrafish melanocytes generated only pigmented nevi. However, injection of the *mitfa:BRAF*<sup>V600E</sup> construct in zebrafish carrying an inactivating mutation in the *tp53* tumour suppressor gene<sup>33</sup>, the human orthologue of which is mutated in approximately 15% of human melanomas, initiated the formation of malignant melanomas in 6% of zebrafish within 4 months<sup>25</sup>. These tumours were confirmed as melanomas by histologic and phenotypic analyses. Importantly, the penetrance of the disease reached nearly 100% in stable *mitfa:BRAF*<sup>V600E</sup>;*tp53*<sup>-/-</sup> F1 transgenic zebrafish. The *mitfa:BRAF*<sup>V600E</sup>;*tp53*<sup>-/-</sup> zebrafish melanoma model has subsequently served as a reliable basis for multiple genetic screens aiming at functionally probing human melanoma

genetics in vivo, and has identified several new modulators of melanoma<sup>34</sup>. Although extremely valuable, this modelling approach posed some technical difficulties. First, like in other well-established transgenic zebrafish cancer models<sup>23,25,35,36</sup>, maintaining stable lines containing several transgenes is difficult. Second, the number of transgenes that can be combined remains limited, which makes it challenging to test complex genetic interactions. Third, unlike overexpression of human oncogenes, knockouts of endogenous zebrafish tumour suppressor genes are systemic, not tissue specific. Finally, most models are still based on transgenic expression of human oncogenes rather than knock-ins into endogenous or orthologue zebrafish loci and thus might not have physiologic levels of expression. Recent advances in genome editing technology have and will help overcome some of these limitations.

### **Nevi**

Benign skin lesions of melanocytes, also known as moles, which are thought to be senescent.

## **Rapid combinatorial genetic modelling**

The CRISPR–Cas9 system created new opportunities for in vivo cancer modelling<sup>37</sup>. It allowed the rapid mosaic inactivation of tumour suppressor genes, bypassing the need for stable transgenic lines. It also greatly increased the potential for combinatorial modelling through multiplexing of guide RNAs (gRNAs) targeting different genes, making possible both the recapitulation of complex human cancer genotypes and the in vivo evaluation of putative genetic interactions inferred from human cancer genomics. Finally, it facilitated large-scale screens with the goal of identifying new cancer drivers.

As in mice, CRISPR made possible the rapid establishment of knockout zebrafish lines<sup>38</sup>. Taking advantage of the *Tol2* transposon technology<sup>39</sup>, which facilitates the insertion of DNA constructs into the zebrafish genome, CRISPR vectors have been developed for tissue-specific gene inactivation by assembling a zebrafish U6 promoter-driven gRNA cassette with a *cas9* sequence placed under the control of tissue-restricted zebrafish promoters<sup>18</sup>. Efficient mosaic gene knockout was observed in embryonic tissues such as muscle and erythroid cells. By designing vectors to target zebrafish tumour suppressor genes, and to express human oncogenes in a melanocyte-specific manner, we were able to rapidly and robustly model all the major genotypes found in human melanoma by combining oncogenic mutations in *BRAF*, *NRAS* or *KIT* with loss-of-function mutations in *cdkn2a*, *tp53*, *ptena* or *ptenb*<sup>12</sup> (FIG. 2a). Tumours were obtained as early as 3 weeks after injection (*NRAS*<sup>Q61R</sup> expression and *tp53* loss) for the most aggressive genotypes or within a few months (*BRAF*<sup>V600E</sup> expression and *cdkn2a* loss), consistent with observations in the human disease<sup>40,41</sup>.

### ***Tol2* transposon**

*Tol2* is an active transposon derived from the medaka fish and is used as a gene delivery method.

### U6 promoter

An rRNA polymerase iii type 3 promoter commonly used to drive short hairpin rNAs or guide rNAs.

This technique thus allows rapid cancer modelling. It is also versatile and could be easily adapted for expression and inactivation of different oncogene and tumour suppressor combinations, respectively, in other organs, provided a robust tissue-restricted zebrafish promoter is available. For example, the Gutierrez group has shown that mutations in the gene *patched 1 (ptch1)* in the Hedgehog pathway increase T-ALL onset using a similar mosaic CRISPR approach<sup>42</sup>. Some genotypes reached 100% penetrance, indicating that mosaic expression of the vectors in the F0 generation was sufficient to generate tumours, which abolishes the need to establish stable transgenic lines for tissues where robust tissue-specific promoters are available. The fact that genetic driver alterations are introduced from birth constitutes a limitation of this approach, as developmental defects may be induced. One way of circumventing this issue is to deliver the vector combinations directly into somatic tissues of adult zebrafish, for example, using a recently reported electroporation method<sup>43</sup>. Finally, this modelling technique is scalable, as several vectors can be injected together to model more complex genotypes. We used this option to study the role of *SPRED1* loss of function in mucosal melanoma, which was inferred from analysing human genomic data, and demonstrated the genetic cooperation between *spred1* inactivation and *KIT* oncogenic mutations in a *tp53*-null or *cdkn2a*-null context<sup>12</sup>. Overexpression and CRISPR vectors can also be multiplexed further to test the relevance of putative cancer drivers in a high-throughput manner. The tools presented above make the zebrafish a prime model to recapitulate and functionally probe the genetics of human tumours in vivo at scale.

### Zebrafish cancer xenografts

**Zebrafish as transplant recipients.**—The zebrafish has many inherent traits that make it an ideal transplant recipient. First, the engrafted normal or malignant cells can be easily tracked by fluorescent labelling of transplanted cells, and direct visualization of grafts is possible using the optically clear casper zebrafish strain as recipients<sup>44</sup>. Second, zebrafish are highly fecund, with matured females capable of producing hundreds of eggs per week, yet are small, so thousands of zebrafish can be kept in a single facility, and thus husbandry and maintenance costs are low relative to those for mice (while this might differ significantly between countries and institutions, it has been reported that the cost ratio is on the order of magnitude of approximately US\$1.05 per mouse versus approximately US\$0.01 per adult zebrafish)<sup>45</sup>. Most importantly, manual transplantation or microinjection can be performed in several hundred adult zebrafish or up to a few thousand zebrafish larvae, respectively, in a single day by a single operator (FIG. 2b), facilitating large-scale and high-throughput cell transplantation studies that are difficult to execute with immunocompromised mouse models<sup>46–53</sup>. Allogeneic transplantation of nonimmune matched zebrafish cells into partially immunocompromised adult zebrafish has now become routine. When paired with large-scale genetic screening and drug discovery platforms available in zebrafish models, such studies have provided valuable insights into intra-tumoural clonal evolution and heterogeneity,

therapy resistance, invasion and metastasis, haematopoiesis and stem cell engraftment<sup>13,47,51,53–57</sup>.

#### Casper zebrafish strain

An optically clear strain of zebrafish obtained by crossing the *roy* (*mpv17*<sup>-/-</sup>) and *nacre* (*mitfa*<sup>-/-</sup>) mutants, which results in the lack of iridophores and melanocytes, respectively.

**Xenografts in zebrafish larvae.**—The xenotransplantation of a human cancer cell line into zebrafish larvae was first described for melanoma in 2006–2007 (REFS<sup>58–60</sup>) (FIG. 1). Since then, a large number of studies have reported xenotransplantation of cancer cell lines into zebrafish larvae. However, especially earlier studies did not always include a rigorous assessment of xenograft survival and proliferation. Ten years later, three proof-of-concept studies were published that developed patient-derived xenografts (PDXs) in zebrafish larvae from both blood malignancies and solid tumours<sup>61–63</sup>, followed by others showing cooperation among tumour-cell subpopulations that can synergize to promote tumour progression<sup>64</sup> and phenotypic testing of drug response<sup>13</sup>.

Adaptive and innate immune systems in zebrafish are highly conserved with mice and humans<sup>65</sup>. In zebrafish, innate immunity starts developing on the first day after fertilization, while adaptive immunity is morphologically and functionally mature only about 2–3 weeks after fertilization<sup>66–69</sup>. The short window of immune incompetency during early larval development has allowed transplantation and short-term survival of xenotransplanted human or mouse cells without the need to immunocompromise the zebrafish. With this approach, numerous cell types have been transplanted into zebrafish, which resulted in many important findings, including mechanisms governing cancer cell differentiation, proliferation and migration, as well as therapeutic response<sup>13,59,70,71</sup>. A particular advantage is the natural optical clarity of wild-type larval zebrafish before they develop pigmentation, which allows facile single-cell resolution imaging, analysis and quantification of transplanted cells, a trait superior to other transplant model organisms.

However, such assays have some inherent limitations. First, larval xenotransplantation experiments have a short window of about 7 days before engrafted cells are rejected by the acquired immune system<sup>13</sup>. Second, the small body size of zebrafish larvae restricts the number of transplanted cells to 100–200 per animal, a number that often fails to contain cancer-driving stem cells or accurately recapitulate the genetic heterogeneity and drug response behaviours driven by rare subclones found in human tumours<sup>13,72</sup>. Third, most xenotransplantation experiments using larval recipients are conducted below 37 °C (REF.<sup>13</sup>), and at those temperatures, transplanted human cells do not proliferate at the same rates or form tumour masses akin to those found in immunocompromised Nsg mice or in human patients<sup>13,14</sup>.

### NSG mice

genetically engineered immunocompromised strain of mice widely used as recipients for engraftment of human primary cells or tumours (for example, patient-derived xenografts).

**Xenografts in adult zebrafish.**—Immunodeficient zebrafish models were recently developed that engraft xenotransplanted patient-derived cancer cells into adult zebrafish recipients<sup>14</sup> (FIG. 1). Previous attempts at facilitating long-term engraftment and survival of xenotransplanted cells in adult zebrafish involved transient ablation of the animal's immune system using radiation or chemical treatment<sup>73,74</sup>. Gamma irradiation of wild-type or casper recipient fish allows robust engraftment of zebrafish tumour and haematopoietic cells for up to 20 days after transplantation<sup>75</sup>, since the host immune system recovers by 30 days after irradiation and rejects the graft, but has not been reported to make possible engraftment of human cells. Long-term chemically induced immunosuppression by constant drug dosing of the steroid drug dexamethasone permits engraftment of a subset of human tumours to time points exceeding 30 days<sup>73</sup>. As dexamethasone itself is a clinically used anticancer drug in leukaemias and lymphomas<sup>76</sup>, this approach would not be suitable for assessing blood and leukaemia cell engraftment. Furthermore, chemical immunosuppression introduces an additional layer of experimental variability and could result in drug interactions with agents tested that might confound experimental result interpretations, thus hindering standardization and scaling.

To overcome these limitations, the Langenau group generated an adult zebrafish xenotransplantation model that allows robust, long-term engraftment and proliferation of human and mouse cells by introducing homozygous inactivating mutations in *prkdc* (which encodes DNA-activated protein kinase catalytic subunit) and *il2rga* (which encodes interleukin-2 receptor- $\gamma$ ) into transparent casper zebrafish<sup>14</sup>. These immunodeficient zebrafish are optically clear, lack T cells, B cells and natural killer cells and can survive at 37 °C. Remarkably, no drawbacks were found in rearing zebrafish at 37 °C, other than additional work in husbandry. Viability is high and zebrafish well tolerate this temperature if acclimated over time. Moreover, acclimated zebrafish robustly engraft a large variety of both human cancer cell lines and PDXs in excess of 28 days<sup>14</sup>. Another major advantage of adult over larval transplant assays is that a significantly larger number of transplanted cells can be used per adult zebrafish (up to two million cells per zebrafish), in addition to the advantages of a longer experimental window and human physiological temperature. Growth kinetics, cell proliferation and apoptotic rates were identical when compared with those for the same tumours grown in NSG mice<sup>14</sup>. Building upon the experience in mouse models<sup>77,78</sup>, we expect that further engineering of the *prkdc*<sup>-/-</sup>*il2rga*<sup>-/-</sup> strain could overcome current limitations, due, for example, to limited conservation between fish and human cytokines, or residual adaptive immunity, and further improve the robustness and increase the range of human tumours engrafted.

## Drug testing in zebrafish avatars

Engrafting a patient's tumour or transgenic modelling with large cohorts of zebrafish permits testing of a wide array of clinically available drugs, and as such pairing responses in zebrafish avatars with clinical decision-making could help stratify patients for the most suitable treatment for their tumour (FIG. 3). The logistic advantages of zebrafish avatars significantly increases the scale of drug testing possible compared with other in vivo patient-specific avatars (TABLE 1).

## Drug administration routes in zebrafish

Zebrafish are treated by adding drugs directly to their water and submersing larvae and adults<sup>12,13</sup>. Furthermore, in adults, drugs can be administered by intraperitoneal injection<sup>79,80</sup> or oral gavage<sup>14,79</sup> (FIG. 3). Drug delivery in larvae via submersion therapy makes it impossible to accurately assess drug dosing, pharmacokinetics and pharmacodynamics (FIG. 3). In contrast, clinically relevant drug administration via oral gavage or intraperitoneal injection in adults allows more tightly controlled dosing and also assessment of pharmacokinetics by mass spectrometry using pooled blood plasma samples. Furthermore, unlike submersion, oral gavage and intraperitoneal injection can effectively deliver small molecules and antibodies that have low solubility in water. Pharmacokinetic differences between humans, mice and zebrafish are an important concern that the field has been confronted with, but from empirical testing have been used effectively to identify dose-conversion factors for oral gavage<sup>14,79</sup> and for submersion therapy<sup>13</sup> (also preliminarily shown in REF.<sup>81</sup>). Furthermore, oral gavage of both a chemotherapy agent (that is, temozolomide) and a targeted therapy (that is, olaparib) in adult zebrafish PDXs (zPDXs) led to a blood pharmacokinetic profile very similar to that in humans and mice<sup>14</sup>. Thus, while optimization will be required for drug dosing on a per case basis, there is robust proof of concept that pharmacokinetics per se is not a cross-species barrier for zebrafish avatars.

## Clinical response determination

Patient clinical response to cancer treatment is determined by measuring maximum tumour diameter changes on imaging data from magnetic resonance imaging or computed tomography scans. On the basis of the size change, the patient's response is classified according to the RECIST criteria<sup>82</sup>. In mouse xenograft tumours, calipers, computed tomography or positron emission tomography, or luciferase bioluminescence is commonly used for tumour response measurement. In zebrafish avatars, drug response has been measured in several ways: (1) direct imaging in transparent recipients of unlabelled tumour cells (particularly for naturally pigmented tumours as melanoma)<sup>79</sup>, (2) fluorescent imaging of tumour size (by either transducing the patient-derived cells with a fluorescent protein viral vector (for example, enhanced GFP), or with FUCCI4 to visualize cell cycle phases)<sup>14</sup>, (3) by exposing the cancer cells to viable fluorescent dyes<sup>13,14</sup> or (4) non-invasively using ultra-sonography<sup>83</sup>. The number of cells (larval zPDX)<sup>13</sup> and tumour surface area (both larval and adult zPDXs)<sup>14</sup> (also preliminarily shown in REF.<sup>81</sup>) have been used as a tumour response measure using RECIST criteria. These methods, combined with the high throughput of in vivo screening offered by the zebrafish platform, provide faster imaging end points that capitalize on the ability to assess drug effects in real time down to single-cell



resolution. The development of automated injector systems (reviewed previously<sup>17</sup>) and the emergence of automated imaging systems for zebrafish larvae<sup>16</sup> further illustrate the high potential for automation and scaling of zPDXs, which would be important for standardization and potential clinical deployment.

#### RECIST criteria

A set of published rules that define when patients with cancer improve ('respond'), stay the same ('stable') or worsen ('progression') during treatments.

#### Types of agents that can be tested

The biological differences between the avatar models highlighted earlier and target conservation in different organisms have immediate implications for what type of therapeutic agents can be assessed effectively (TABLE 1). Many anticancer agents have been successfully used in zebrafish, including various types of chemotherapy, small-molecule inhibitors such as dasatinib and antibody-based therapies such as bevacizumab and cetuximab<sup>13,62,79,84–94</sup>. For example, the adult zPDX model was used to identify the combination of the poly(ADP-ribose) polymerase (PARP) inhibitor olaparib and the chemotherapy agent temozolomide as an effective therapy for rhabdomyosarcoma. The combination treatment efficacy was recapitulated in a mouse xenograft model, giving further proof of the equivalency of the two recipient model organisms for drug testing<sup>14</sup>. This drug combination is now entering clinical trials for paediatric rhabdomyosarcoma (NCT01858168)<sup>95</sup> and represents the first clinical trial of a combination therapy initiated from xenograft preclinical studies using zebrafish. Chemotherapy has a high degree of conservation of targets across zebrafish and humans, while targeted therapies using small molecules or antibodies are highly dependent on the target's structural conservation for its action. Thus, intrinsic targets in cancer cells can usually be assessed effectively only in xenograft cell transplant models<sup>62,79,84–92</sup>. On the other hand, targeted therapy directed to the tumour microenvironment (TME) will present a more variable degree of feasibility depending on exact target conservation. Similar considerations apply to transgenic models where we typically introduce human versions of driver oncogenes<sup>12,34,79</sup>, but the success of therapies targeting other genes or synthetic lethal approaches will differ depending on target conservation<sup>20,96,97</sup>. As in engraftment studies using NSG mice, the lack of a patient-specific immune tumour microenvironment and/or co-evolution in zPDX models, and lack of cross-species target conservation (for example, limited sequence conservation of immune checkpoints such as PD1, PDL1 and CTLA4) poses challenges to providing an effective proxy in zebrafish avatars to assess patient responses to agents that target the immune TME. Another confounding factor specifically in larvae is the potential toxic effects of many anticancer agents on the zebrafish recipient larva itself, as it goes through a rapid phase of development. Indeed an observed tumour shrinkage might also be the result of an indirect effect of the toxicity on the recipient itself that would compromise its ability to support cancer cell growth<sup>98–103</sup>.

## Prediction of response and resistance

The value of zebrafish avatars for precision cancer therapy boils down to (1) the predictive power, (2) the scale of experimentation and (3) the assay duration time to inform clinical decision-making. Thus, it is worth considering the benefit and limitations pertaining to the time and the effectiveness of zebrafish avatars in predicting not just drug sensitivity but also drug resistance. Drug resistance is often classified as primary or intrinsic resistance (when patients never respond to an agent) and secondary or acquired resistance (when a patient initially sensitive to the drug develops resistance). The former is often determined by the genetic make-up of the tumour's dominant clones or the TME, while the latter has been proposed to be acquired as a result of Darwinian selection of minor genetically resistant pre-existing clones or transcriptional Lamarckian adaptation followed by genetic fixation of a trait<sup>104</sup>.

As an example, primary genetic resistance was probed in zebrafish by rapid combinatorial transgenic modelling of *spred1* inactivation in the context of BRAF- or KIT-driven melanoma<sup>12</sup>. Treatment of adult transgenic zebrafish by submersion with targeted therapies for patients with mucosal melanoma showed this prevalent loss-of-function genetic event drives resistance to drugs inhibiting KIT tyrosine kinase activity<sup>12</sup> (FIG. 3). Mechanistic studies further suggested the potential efficacy of combinations of MEK and KIT inhibitors<sup>12</sup>. Given the short duration of drug exposure required to identify primary resistance, both larval and adult zPDXs are well-suited models to address this question<sup>13</sup>. Larval zPDXs developed from tumour biopsy samples, much like tumour organoids<sup>105</sup>, might be particularly useful in the neoadjuvant setting before surgery to help rapidly pick an effective chemotherapy regimen.

On the other hand, such a short-term larval transplant assay with a limited number of cells per recipient is unlikely to be a good predictor of secondary resistance. This was evident in the work of Fior et al.<sup>13</sup> mimicking a polyclonal tumour, where two isogenic cell lines (HCT116 cells with mutant *KRAS* and Hke3 cells with wild-type *KRAS*) were mixed 1:1 before transplantation, each labelled with a different fluorescent colour. While exposure of the polyclonal tumour to the folinic acid–5-fluorouracil–oxaliplatin (FOLFOX) chemotherapy regimen caused the tumour to shrink overall, the Hke3 resistant clone did not change in size<sup>13</sup>. Thus, without the differential colour labeling, an actual tumour would score overall as sensitive even if a very sizeable subclone failed to respond<sup>106</sup>. It is reasonable to speculate that the longer time window and the larger number of cells of adult zPDXs would better position these models to predict secondary resistance mechanisms based on expansion of a minor genetically resistant clone or allow the time required for transcriptional adaptation. Overall, the ability of these avatars to accurately model and predict resistance will be crucial for their translation to the clinic.

## Comparison between cancer avatars

### Zebrafish and mouse transgenic models

Different cancer avatars have different advantages and limitations, both biologically and logistically, that render their use more suitable to either preclinical drug development or

personalized drug testing. In the case of transgenic cancer models in general, the key factors limiting their application in precision oncology are the time required to generate the avatar and the scale of combinatorial genetics to approximate genomic cancer complexity. For a comparison of zebrafish, mouse and organoid cancer avatar models, see TABLE 1.

Owing to recent advances in cloning and DNA synthesis methods, zebrafish transgenesis vectors can be generated in as little as 1–2 weeks. The time required to establish a transgenic mosaic tumour model in zebrafish ranges from 10 days to 8 weeks in early-onset models such as human *KRAS*<sup>G12D</sup>-driven rhabdomyosarcoma<sup>36</sup>, mouse *Myc*-driven T-ALL<sup>107</sup>, *NRAS*<sup>Q61K</sup>-driven melanoma<sup>12,31</sup> or melanoma resulting from localized skin electroporation of a CRISPR vector targeting *rb1* (which encodes retinoblastoma 1) in adult *BRAF*<sup>V600E</sup>;*tp53*<sup>-/-</sup> zebrafish<sup>43</sup>. Other transgenic models may take many months to develop, such as sarcoma models (for example, rhabdomyosarcoma driven by the *PAX3*–*FOXO1* translocation under the control of the CMV promoter has 16% penetrance at 19 months)<sup>35</sup> (FIG. 2). To model genomic complexity, combination of three to four genomic events has been successfully achieved, with a possibility to further scale up to ~10–20 alterations depending on limitations of pooled vector transgenesis approaches<sup>12,18</sup>. Strategies for expressing arrays of gRNAs from a single vector might further expand this limit for CRISPR knockout strategies<sup>108,109</sup>.

The speed and scalability of combinatorial genetics of zebrafish mosaic transgenic models far surpass those of most genetically engineered mouse models by avoiding the need to perform multigenerational crosses<sup>110</sup>. The small number of mouse models that leverage in vivo mosaic transgene delivery of plasmid DNA using hydrodynamic injection (liver cancer)<sup>111</sup> or viral vectors (lung cancer)<sup>112</sup> have similar capabilities to zebrafish models in terms of the time to tumour onset and combinatorial genetics. However, the challenges and cost of housing for mice, biosafety, vector production and handling pose a much greater barrier to the generation of a large enough number of animals for testing multiple therapeutic agents. In comparison, a single operator can perform a few thousand zebrafish embryo microinjections in a day to generate genetic patient avatars using only picograms to nanograms of plasmid DNA under biosafety level 1 conditions. Furthermore, the husbandry space requirements (12 adult zebrafish versus 1 adult mouse per ~1.2 l) and cost (of US cents per 3.5-l fish tank versus US dollars per mouse cage), while variable across countries and institutions, are at least an order of magnitude lower for zebrafish than for mice<sup>113</sup>.

#### Hydrodynamic injection

rapid injection of a relatively large volume of DNA solution, which is used for gene delivery in mouse livers.

Mosaic transgenic zebrafish cancer models remain most useful today as tools for preclinical drug development of genotype-stratified targeted therapies, and for phenotypic dissection of genetic events identified in sequencing studies (for example, identification of genetic drivers of drug resistance, functional validation of a gene's role or identification of novel combination treatments to overcome resistance). It is also important to note that both zebrafish and mouse transgenic models have limited genetic heterogeneity, often do not

recapitulate the sequential order of tumorigenic events seen in patients and have limited capabilities of modelling subclonal architecture<sup>12,34,43</sup>. Thus, modelling intra-tumour heterogeneity still presents major challenges and remains a barrier for these models to predict drug responses in patients.

### PDXs and human organoids

Approaches using patient-derived tumour material for empirical drug testing are limited by several factors: (1) the ability to culture in vitro or successfully engraft in vivo patient-derived cancer cells in a recipient animal model; (2) the finite number of cells obtained from biopsy or surgical resection that can be used for in vitro culture or in vivo xenotransplantation; (3) the time between the biopsy and the readout of the assay; and (4) the degree of fidelity to which these models maintain representation of the tumour heterogeneity and clonal evolution within the patient, and also its microenvironment and co-evolution with the patient's immune system.

With regard to the first point, xenotransplantation of patient-derived cancer cells into zebrafish has been reported to have a high degree of success in proof-of-concept studies, both in the successful establishment of zPDXs from a specific patient and in the proportion of recipient zebrafish showing engraftment from a specific donor. Fior et al.<sup>13</sup> reported 100% donor engraftment in a small clinical study of patients with colorectal cancer ( $n = 5$ ), with 47–89% of zebrafish larval recipients ( $n = 47$ –251) showing engraftment. Preliminary partial results of clinical study [NCT03668418](#) (REF.<sup>114</sup>) conducted as a co-clinical trial involving 24 adult patients with pancreatic cancer ( $n = 12$ ), colon cancer ( $n = 8$ ) or gastric cancer ( $n = 4$ ) similarly suggested engraftment of all donors, with comparable survival of recipient zebrafish larvae<sup>81</sup>. Other studies reported successful engraftment in larvae of PDXs from gastric cancer<sup>86</sup>, adenoid cystic carcinoma<sup>85</sup> and pancreatic cancer<sup>87</sup>. Similarly, xenotransplantation in *prkdc*<sup>-/-</sup>, *il2rga*<sup>-/-</sup> recipient adult zebrafish at 37 °C of six PDXs from patients with melanoma ( $n = 2$ ), breast cancer ( $n = 1$ ), glioma ( $n = 1$ ) or embryonal rhabdomyosarcoma ( $n = 2$ ) resulted in engraftment of all six donors, with 35–80% of recipient animals showing engraftment (four to eight transplant recipients per donor patient)<sup>14</sup>.

Second, the number of cells used per recipient is both a limitation and an advantage. Patient material is a finite and often non-renewable resource, especially if it comes from a biopsy or the excision of a small tumour. Thus, organoids or zebrafish transplantation in larvae is advantageous since it requires only ~100 cells per well or recipient. Thus, even small quantities of patient-derived cells can be used effectively to test many conditions, drugs, dosing regimens or drug combinations. Xenotransplantation in adult zebrafish or mice, on the other hand, requires  $10^5$  or  $10^5$ – $10^6$  cells, respectively<sup>5,14</sup>.

Third, organoid-based drug assays require 1–2 weeks<sup>105,115–117</sup>, zPDXs in larvae require 4–7 days<sup>13</sup> and adult zebrafish and mouse PDXs require many weeks or even months to read out. On the other hand, the higher number of cells and the longer time window in adult zebrafish and mouse xenografts allows us to observe and perhaps model the effects of clonal evolution and adaptation, which has implications for drug resistance prediction discussed further below.

Some limitations inherent to transplantation-based avatars are common between mouse and zebrafish PDXs. Mouse studies highlight how the engraftment bottlenecks and evolution in a recipient animal might alter patient representation or result in evolution patterns that are discordant between patient and mouse avatars<sup>118</sup>. Krivtsov and colleagues<sup>119</sup> reported a 50% clonal discordance in mouse PDXs of leukaemia when analysing genetic driver allele frequency, and Golub and colleagues<sup>120</sup> showed mouse-specific tumour evolution of a large number of PDXs across 24 cancer types. On the other hand, human organoids seem to maintain genetic representation of the patient source material during short-term in vitro culture<sup>115</sup>. Large-scale comparative studies will be needed to determine the degree to which genetic and transcriptional heterogeneity is preserved in adult zPDX avatars over time.

While clinical prediction studies have been limited in frequency and scale due to the young age of the field, the results of several proof-of-concept studies have been encouraging. Fior et al.<sup>13</sup> tested the response to FOLFOX chemotherapy and cetuximab using five colorectal cancer PDXs in zebrafish larvae. Concordant results were obtained between zPDXs and actual patient clinical responses for FOLFOX (four of five patients) and cetuximab (three of three patients)<sup>13</sup>. While these numbers are obviously small, for comparison, the largest prospective clinical study to date on the predictive value of human organoids for treatment response enrolled 61 patients with colorectal cancer and reported a 63% success rate in establishing in vitro cultures from patient tissue, with only 29 of 61 patients (~47%) with an evaluable response and an 80% prediction rate<sup>121</sup>. This study thus highlights both the promise and also the challenges of phenotypic testing of drug response even in technologies more mature than zebrafish avatars. In addition to the intrinsic heterogeneity of cancer cells, an ever-growing body of evidence has shown the importance of other cell types present in the local TME in modulating therapy response<sup>122,123</sup>. Much like mouse PDXs, the contribution of local TME components in zebrafish xenotransplantation is limited, and host-systemic effects such as immune co-evolution and systemic endocrine conditioning are not captured by transplantation-based zebrafish avatars (TABLE 1). This structural constraint of all transplant-based but more broadly patient-derived tumour models limits the predictive power of all of these assays in predicting drug sensitivity.

## Concluding remarks

Recent advances showing organoid fidelity in mimicking clinical tumour behaviours<sup>105,115–117</sup> and the first report of a prospective clinical trial for organoids as predictors of patient outcome<sup>121</sup> provided a proof of concept for the clinical value of phenotypic testing of drug response at large. Yet, the very same prospective study<sup>121</sup> highlighted how narrow the predictive power of these in vitro tools still is. Similarly, since they were proposed in 2011, mouse co-clinical trials using PDXs have shown little progress beyond proof of concept due to logistical challenges<sup>5,124,125</sup>. Zebrafish cancer avatars offer unique advantages over current in vivo models in scale, cost and speed of model development and potential for automation.

In terms of personalized transgenic avatars, the time barriers (1–4 weeks for sequencing, data analysis and vector generation, plus weeks to months of tumour latency time) limit their benefit to inform clinical decision-making. Thus, they remain most useful as preclinical

models to unravel cancer genetics and for drug discovery. Continued reductions in the cost and increases in the speed and adoption of both DNA sequencing and synthesis, together with improvements in genome engineering technologies, should allow the generation of ever-faster and more complex transgenic zebrafish models for profiling likely therapeutic responses of individual patients. These studies should benefit precision cancer medicine both by directly affecting the specific patients modelled and also by increasing our understanding of cancer genomics more generally. By functionally assessing the effect of specific mutations on drug response modulation, they should also increase our ability to formulate response predictions based on inferential models analysing the alterations observed in tumour biopsy samples from various types of omics profiling.

On the other hand, now that the technology for zebrafish xenograft cell transplantation has matured, and robust proof-of-concept studies have been provided for the generation of both larval and adult zPDXs, it is paramount for the field to move towards clinical deployment and to conduct clinical studies to assess their predictive power and value in informing clinical decisions. A first ongoing trial (NCT03668418)<sup>114</sup> aiming to enrol 120 patients with cancer to assess the predictive power of larval zPDXs is a good example in this direction and will provide useful insights into the value of larval zPDXs overall. It is still unclear whether zPDXs in larvae can provide significant advantages compared with human organoids, and head-to-head studies will be needed to address this question. These larval models could be of value in clinical settings where speed and short-term responses are the goal, such as presurgery neoadjuvant chemotherapy. Beyond drug response prediction, assessment of metastatic behaviour has been proposed using zebrafish PDXs in larvae<sup>13</sup>, but further studies are needed to assess whether migration in the developing embryo is informative of metastatic risk or organ tropism of human cancer. Evidence of substantial biological and predictive equivalency between mouse PDXs and adult zPDXs, combined with the lower cost and husbandry logistic footprint, and higher throughput of drug screening of the zebrafish model would render it an attractive alternative for precision cancer therapy (TABLE 1). Ultimately cost-benefit analyses will guide which players in the phenotypic drug-testing field will move forward in the clinic. A high predictive power will be needed as motivation for the cost-intensive establishment and operation of dedicated facilities necessary for any avatar model. The potential clinical benefit to patients in terms of survival outcome and spared toxic effects, and the potential cost saving from avoiding wasteful and dangerous administration of ineffective treatments, are an unmet need that the further development of personalized cancer avatars strives to address.

## Acknowledgements

L.I.Z. is supported by the Melanoma Research Alliance and US National Institutes of Health (grants P01CA163222 and R01 CA103846). M.F. was supported by Boehringer Ingelheim Fonds. D.M.L. is funded by the US National Institutes of Health (grants R01CA154923, R01CA211734, R01CA215118, R01CA226926 and R24OD016761) and the MGH Research Scholars Program. Y.C. is supported by the Alex Lemonade Stand Foundation.

## References

1. National Cancer Institute. Targeted cancer therapies. NCI <https://www.cancer.gov/about-cancer/treatment/types/targeted-therapies/targeted-therapies-fact-sheet> (2020).

2. Morrison C Fresh from the biotech pipeline — 2018. *Nat. Biotechnol* 37, 118–123 (2019). [PubMed: 30718867]
3. Friedman AA, Letai A, Fisher DE & Flaherty KT Precision medicine for cancer with next-generation functional diagnostics. *Nat. Rev. Cancer* 15, 747–756 (2015). [PubMed: 26536825]
4. Hyman DM, Taylor BS & Baselga J Implementing genome-driven oncology. *Cell* 168, 584–599 (2017). [PubMed: 28187282]
5. Clohessy JG & Pandolfi PP Mouse hospital and co-clinical trial project — from bench to bedside. *Nat. Rev. Clin. Oncol* 12, 491 (2015). [PubMed: 25895610] This review discusses mouse co-clinical trials for precision cancer therapy.
6. Drost J & Clevers H Organoids in cancer research. *Nat. Rev. Cancer* 18, 407–418 (2018). [PubMed: 29692415]
7. Tuveson D & Clevers H Cancer modeling meets human organoid technology. *Science* 364, 952–955 (2019). [PubMed: 31171691]
8. Schwartzberg L, Kim ES, Liu D & Schrag D Precision oncology: who, how, what, when, and when not? *Am. Soc. Clin. Oncol. Educ. Book* 37, 160–169 (2017). [PubMed: 28561651]
9. Strauss DG & Blinova K Clinical trials in a dish. *Trends Pharmacol. Sci* 38, 4–7 (2017). [PubMed: 27876286]
10. Aparicio S, Hidalgo M & Kung AL Examining the utility of patient-derived xenograft mouse models. *Nat. Rev. Cancer* 15, 311 (2015). [PubMed: 25907221]
11. Tentler JJ et al. Patient-derived tumour xenografts as models for oncology drug development. *Nat. Rev. Clin. Oncol* 9, 338 (2012). [PubMed: 22508028]
12. Ablain J et al. Human tumor genomics and zebrafish modeling identify SPRED1 loss as a driver of mucosal melanoma. *Science* 362, 1055–1060 (2018). [PubMed: 30385465] This article shows rapid generation of genetic avatars in zebrafish using combinatorial mosaic transgenesis.
13. Fior R et al. Single-cell functional and chemosensitive profiling of combinatorial colorectal therapy in zebrafish xenografts. *Proc. Natl Acad. Sci. USA* 114, E8234–E8243 (2017). [PubMed: 28835536] This study conducts phenotypic drug response testing and migration scoring of PDXs in zebrafish larvae.
14. Yan C et al. Visualizing engrafted human cancer and therapy responses in immunodeficient zebrafish. *Cell* 177, 1903–1914 (2019). [PubMed: 31031007] This article demonstrates establishment of PDXs in adult zebrafish.
15. White R, Rose K & Zon L Zebrafish cancer: the state of the art and the path forward. *Nat. Rev. Cancer* 13, 624–636 (2013). [PubMed: 23969693]
16. Pulak R Tools for automating the imaging of zebrafish larvae. *Methods* 96, 118–126 (2016). [PubMed: 26631716]
17. Zhao Y et al. A review of automated microinjection of zebrafish embryos. *Micromachines* 10, 7 (2018).
18. Ablain J, Durand EM, Yang S, Zhou Y & Zon LI A CRISPR/Cas9 vector system for tissue-specific gene disruption in zebrafish. *Dev. Cell* 32, 756–764 (2015). [PubMed: 25752963]
19. Gengenbacher N, Singhal M & Augustin HG Preclinical mouse solid tumour models: status quo, challenges and perspectives. *Nat. Rev. Cancer* 17, 751 (2017). [PubMed: 29077691]
20. Howe K et al. The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496, 498–503 (2013). [PubMed: 23594743]
21. Carneiro MC, de Castro IP & Ferreira MG Telomeres in aging and disease: lessons from zebrafish. *Dis. Model. Mech* 9, 737–748 (2016). [PubMed: 27482813]
22. Berghmans S et al. Making waves in cancer research: new models in the zebrafish. *Biotechniques* 39, 227–237 (2005). [PubMed: 16116796]
23. Langenau DM et al. Myc-induced T cell leukemia in transgenic zebrafish. *Science* 299, 887–890 (2003). [PubMed: 12574629] This article reports the first transgenic cancer model in zebrafish.
24. Yang HW et al. Targeted expression of human MYCN selectively causes pancreatic neuroendocrine tumors in transgenic zebrafish. *Cancer Res.* 64, 7256–7262 (2004). [PubMed: 15492244]

25. Patton EE et al. BRAF mutations are sufficient to promote nevi formation and cooperate with p53 in the genesis of melanoma. *Curr. Biol* 15, 249–254 (2005). [PubMed: 15694309]
26. Cagan RL, Zon LI & White RM Modeling cancer with flies and fish. *Dev. Cell* 49, 317–324 (2019). [PubMed: 31063751]
27. Kirchberger S, Sturtzel C, Pascoal S & Distel M *Quo natus, Danio?*— Recent progress in modeling cancer in zebrafish. *Front. Oncol* 7, 186 (2017). [PubMed: 28894696]
28. Letrado P, de Miguel I, Lamberto I, Diez-Martinez R & Oyarzabal J Zebrafish: speeding up the cancer drug discovery process. *Cancer Res.* 78, 6048–6058 (2018). [PubMed: 30327381]
29. Vogelstein B et al. Cancer genome landscapes. *Science* 339, 1546–1558 (2013). [PubMed: 23539594]
30. Vogelstein B & Kinzler KW The path to cancer – three strikes and you’re out. *N. Engl. J. Med* 373, 1895–1898 (2015). [PubMed: 26559569]
31. McConnell AM et al. Neural crest state activation in NRAS driven melanoma, but not in NRAS-driven melanocyte expansion. *Dev. Biol* 449, 107–114 (2019). [PubMed: 29883661]
32. Pea A, Hruban RH & Wood LD Genetics of pancreatic neuroendocrine tumors: implications for the clinic. *Expert Rev. Gastroenterol. Hepatol* 9, 1407–1419 (2015). [PubMed: 26413978]
33. Berghmans S et al. tp53 mutant zebrafish develop malignant peripheral nerve sheath tumors. *Proc. Natl Acad. Sci. USA* 102, 407–412 (2005). [PubMed: 15630097]
34. Ceol CJ et al. The histone methyltransferase SETDB1 is recurrently amplified in melanoma and accelerates its onset. *Nature* 471, 513–517 (2011). [PubMed: 21430779]
35. Kendall GC et al. PAX3- FOXO1 transgenic zebrafish models identify HES3 as a mediator of rhabdomyosarcoma tumorigenesis. *eLife* 7, e33800 (2018). [PubMed: 29869612]
36. Langenau DM et al. Effects of RAS on the genesis of embryonal rhabdomyosarcoma. *Genes Dev.* 21, 1382–1395 (2007). [PubMed: 17510286]
37. Sanchez-Rivera FJ & Jacks T Applications of the CRISPR-Cas9 system in cancer biology. *Nat. Rev. Cancer* 15, 387–395 (2015). [PubMed: 26040603]
38. Hwang WY et al. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat. Biotechnol* 31, 227–229 (2013). [PubMed: 23360964] This study reports the first use of CRISPR in zebrafish.
39. Kawakami K et al. A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish. *Dev. Cell* 7, 133–144 (2004). [PubMed: 15239961]
40. Heppt MV et al. Prognostic significance of BRAF and NRAS mutations in melanoma: a German study from routine care. *BMC Cancer* 17, 536 (2017). [PubMed: 28797232]
41. Shain AH et al. The genetic evolution of melanoma from precursor lesions. *N. Engl. J. Med* 373, 1926–1936 (2015). [PubMed: 26559571]
42. Burns MA et al. Hedgehog pathway mutations drive oncogenic transformation in high-risk T-cell acute lymphoblastic leukemia. *Leukemia* 32, 2126–2137 (2018). [PubMed: 29654263]
43. Callahan SJ et al. Cancer modeling by transgene electroporation in adult zebrafish (TEAZ). *Dis. Model. Mech* 11, (2018). This article shows mosaic transgenesis in adult zebrafish through electroporation of plasmid DNA.
44. White RM et al. Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell Stem Cell* 2, 183–189 (2008). [PubMed: 18371439] This article reports development of the optically clear casper zebrafish strain often used for transplantation studies.
45. Weintraub A All eyes on zebrafish. *Lab. Anim* 46, 323–326 (2017).
46. Smith AC et al. High-throughput cell transplantation establishes that tumor-initiating cells are abundant in zebrafish T-cell acute lymphoblastic leukemia. *Blood* 115, 3296–3303 (2010). [PubMed: 20056790]
47. Blackburn JS et al. Clonal evolution enhances leukemia-propagating cell frequency in T cell acute lymphoblastic leukemia through Akt/mTORC1 pathway activation. *Cancer Cell* 25, 366–378 (2014). [PubMed: 24613413]
48. Tang Q et al. Optimized cell transplantation using adult rag2 mutant zebrafish. *Nat. Methods* 11, 821–824 (2014). [PubMed: 25042784] This article reports allotransplantation of zebrafish tumours in immunocompromised recipients.

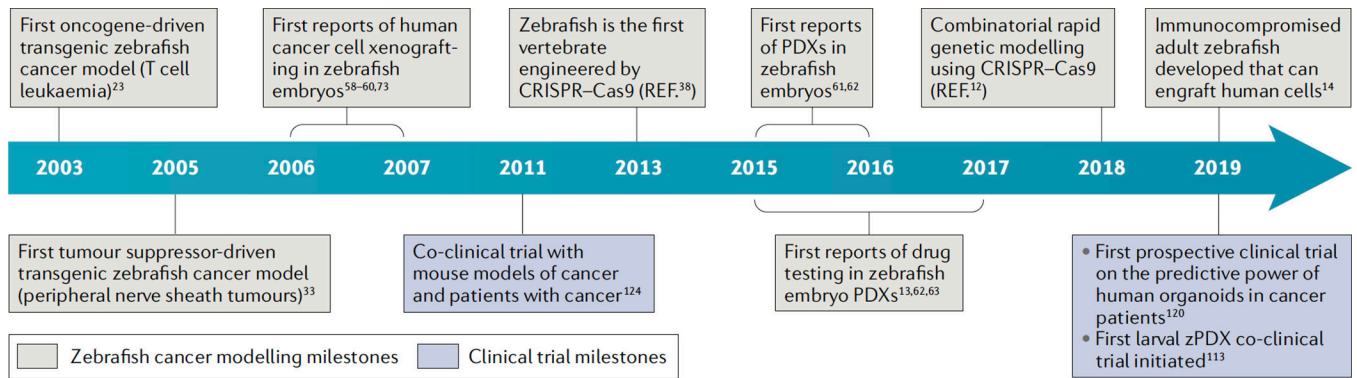


49. Tang Q et al. Imaging tumour cell heterogeneity following cell transplantation into optically clear immune-deficient zebrafish. *Nat. Commun* 7, 10358 (2016). [PubMed: 26790525]
50. Hayes MN et al. Vangl2/RhoA signaling pathway regulates stem cell self-renewal programs and growth in rhabdomyosarcoma. *Cell Stem Cell* 22, 414–427.e6 (2018). [PubMed: 29499154]
51. Ignatius MS et al. The NOTCH1/SNAIL1/MEF2C pathway regulates growth and self-renewal in embryonal rhabdomyosarcoma. *Cell Rep.* 19, 2304–2318 (2017). [PubMed: 28614716]
52. Moore JC et al. Single-cell imaging of normal and malignant cell engraftment into optically clear prkdc-null SCID zebrafish. *J. Exp. Med* 213, 2575–2589 (2016). [PubMed: 27810924]
53. Tenente IM et al. Myogenic regulatory transcription factors regulate growth in rhabdomyosarcoma. *eLife* 6, e19214 (2017). [PubMed: 28080960]
54. Li P et al. Epoxyeicosatrienoic acids enhance embryonic haematopoiesis and adult marrow engraftment. *Nature* 523, 468–471 (2015). [PubMed: 26201599]
55. Tamplin OJ et al. Hematopoietic stem cell arrival triggers dynamic remodeling of the perivascular niche. *Cell* 160, 241–252 (2015). [PubMed: 25594182]
56. Heilmann S et al. A quantitative system for studying metastasis using transparent zebrafish. *Cancer Res.* 75, 4272–4282 (2015). [PubMed: 26282170]
57. Zhang M et al. Adipocyte-derived lipids mediate melanoma progression via FATP proteins. *Cancer Discov.* 8, 1006–1025 (2018). [PubMed: 29903879]
58. Nicoli S, Ribatti D, Cotelli F & Presta M Mammalian tumor xenografts induce neovascularization in zebrafish embryos. *Cancer Res.* 67, 2927–2931 (2007). [PubMed: 17409396]
59. Haldi M, Ton C, Seng WL & McGrath P Human melanoma cells transplanted into zebrafish proliferate, migrate, produce melanin, form masses and stimulate angiogenesis in zebrafish. *Angiogenesis* 9, 139–151 (2006). [PubMed: 17051341]
60. Topczewska JM et al. Embryonic and tumorigenic pathways converge via Nodal signaling: role in melanoma aggressiveness. *Nat. Med* 12, 925–932 (2006). [PubMed: 16892036]
61. Mercatali L et al. Development of a patient-derived xenograft (PDX) of breast cancer bone metastasis in a zebrafish model. *Int. J. Mol. Sci* 17, 1375 (2016).
62. Bentley VL et al. Focused chemical genomics using zebrafish xenotransplantation as a pre-clinical therapeutic platform for T-cell acute lymphoblastic leukemia. *Haematologica* 100, 70–76 (2015). [PubMed: 25281505]
63. Lin J et al. A clinically relevant in vivo zebrafish model of human multiple myeloma to study preclinical therapeutic efficacy. *Blood* 128, 249–252 (2016). [PubMed: 27207793]
64. Chapman A et al. Heterogeneous tumor subpopulations cooperate to drive invasion. *Cell Rep.* 8, 688–695 (2014). [PubMed: 25066122]
65. Renshaw SA & Trede NS A model 450 million years in the making: zebrafish and vertebrate immunity. *Dis. Model. Mech* 5, 38–47 (2012). [PubMed: 22228790]
66. Langenau DM et al. In vivo tracking of T cell development, ablation, and engraftment in transgenic zebrafish. *Proc. Natl Acad. Sci. USA* 101, 7369–7374 (2004). [PubMed: 15123839]
67. Tang Q et al. Dissecting hematopoietic and renal cell heterogeneity in adult zebrafish at single-cell resolution using RNA sequencing. *J. Exp. Med* 214, 2875–2887 (2017). [PubMed: 28878000]
68. Trede NS, Langenau DM, Traver D, Look AT & Zon LI The use of zebrafish to understand immunity. *Immunity* 20, 367–379 (2004). [PubMed: 15084267]
69. Willett CE, Cortes A, Zuasti A & Zapata AG Early hematopoiesis and developing lymphoid organs in the zebrafish. *Dev. Dyn* 214, 323–336 (1999). [PubMed: 10213388]
70. Konantz M et al. Zebrafish xenografts as a tool for in vivo studies on human cancer. *Ann. NY Acad. Sci* 1266, 124–137 (2012). [PubMed: 22901264]
71. He S et al. Neutrophil-mediated experimental metastasis is enhanced by VEGFR inhibition in a zebrafish xenograft model. *J. Pathol* 227, 431–445 (2012). [PubMed: 22374800]
72. Visvader JE & Lindeman GJ Cancer stem cells: current status and evolving complexities. *Cell Stem Cell* 10, 717–728 (2012). [PubMed: 22704512]
73. Stoletov K, Montel V, Lester RD, Gonias SL & Klemke R High-resolution imaging of the dynamic tumor cell vascular interface in transparent zebrafish. *Proc. Natl Acad. Sci. USA* 104, 17406–17411 (2007). [PubMed: 17954920]

74. Traver D et al. Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. *Nat. Immunol* 4, 1238–1246 (2003). [PubMed: 14608381]
75. Traver D et al. Effects of lethal irradiation in zebrafish and rescue by hematopoietic cell transplantation. *Blood* 104, 1298–1305 (2004). [PubMed: 15142873]
76. Larsen EC et al. Dexamethasone and high-dose methotrexate improve outcome for children and young adults with high-risk B-acute lymphoblastic leukemia: a report from Children’s Oncology Group Study AALL0232. *J. Clin. Oncol* 34, 2380–2388 (2016). [PubMed: 27114587]
77. Matsuda M et al. Human NK cell development in hIL-7 and hIL-15 knockin NOD/SCID/IL2rgKO mice. *Life Sci. Alliance* 2, 201800195 (2019).
78. Herndler-Brandstetter D et al. Humanized mouse model supports development, function, and tissue residency of human natural killer cells. *Proc. Natl Acad. Sci. USA* 114, E9626–E9634 (2017). [PubMed: 29078283] This article reports engineering of NSG mice towards humanized models and more robust PDX support.
79. Dang M, Henderson RE, Garraway LA & Zon LI Long-term drug administration in the adult zebrafish using oral gavage for cancer preclinical studies. *Dis. Model. Mech* 9, 811–820 (2016). [PubMed: 27482819] This study administers a drug in adult zebrafish through intraperitoneal injection and oral gavage.
80. Samaee SM, Seyedin S & Varga ZM An affordable intraperitoneal injection setup for juvenile and adult zebrafish. *Zebrafish* 14, 77–79 (2017). [PubMed: 27841973]
81. Usai A et al. A model of zebrafish avatar for co-clinical trials. *Cancers* 12, 677 (2019). This article reports preliminary results of zebrafish co-clinical trial [NCT03668418](https://clinicaltrials.gov/ct2/show/NCT03668418).
82. Eisenhauer EA et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur. J. Cancer* 45, 228–247 (2009). [PubMed: 19097774]
83. Goessling W, North TE & Zon LI Ultrasound biomicroscopy permits in vivo characterization of zebrafish liver tumors. *Nat. Methods* 4, 551–553 (2007). [PubMed: 17572681]
84. Jin Y et al. Comparison of efficacy and toxicity of bevacizumab, endostar and apatinib in transgenic and human lung cancer xenograft zebrafish model. *Sci. Rep* 8, 15837 (2018). [PubMed: 30367145]
85. Chen C et al. A multiplex preclinical model for adenoid cystic carcinoma of the salivary gland identifies regorafenib as a potential therapeutic drug. *Sci. Rep* 7, 11410 (2017). [PubMed: 28900283]
86. Wu JQ et al. Patient-derived xenograft in zebrafish embryos: a new platform for translational research in gastric cancer. *J. Exp. Clin. Cancer Res* 36, 160 (2017). [PubMed: 29141689]
87. Wang L et al. Patient-derived heterogeneous xenograft model of pancreatic cancer using zebrafish larvae as hosts for comparative drug assessment. *J. Vis. Exp* 146, e59507 (2019). This is a method guide for the drug testing of zPDXs in larvae.
88. Ikonomopoulou MP et al. Gimesin inhibits melanoma growth by manipulating key signaling cascades that control cell death and proliferation. *Sci. Rep* 8, 11519 (2018). [PubMed: 30068931]
89. Wang G et al. The novel autophagy inhibitor elaiophylin exerts antitumor activity against multiple myeloma with mutant TP53 in part through endoplasmic reticulum stress-induced apoptosis. *Cancer Biol. Ther* 18, 584–595 (2017). [PubMed: 28718729]
90. von Massenhausen A et al. Targeting DDR2 in head and neck squamous cell carcinoma with dasatinib. *Int. J. Cancer* 139, 2359–2369 (2016). [PubMed: 27434411]
91. Ochoa-Alvarez JA et al. Antibody and lectin target podoplanin to inhibit oral squamous carcinoma cell migration and viability by distinct mechanisms. *Oncotarget* 6, 9045–9060 (2015). [PubMed: 25826087]
92. Ghotra VP et al. SYK is a candidate kinase target for the treatment of advanced prostate cancer. *Cancer Res.* 75, 230–240 (2015). [PubMed: 25388286]
93. van der Ent W et al. Ewing sarcoma inhibition by disruption of EWSR1-FLI1 transcriptional activity and reactivation of p53. *J. Pathol* 233, 415–424 (2014). [PubMed: 24974828]
94. Tan DS et al. Bosutinib inhibits migration and invasion via ACK1 in KRAS mutant non-small cell lung cancer. *Mol. Cancer* 13, 13 (2014). [PubMed: 24461128]
95. US National Library of Medicine. [ClinicalTrials.gov https://clinicaltrials.gov/ct2/show/NCT01858168](https://clinicaltrials.gov/ct2/show/NCT01858168) (2013).

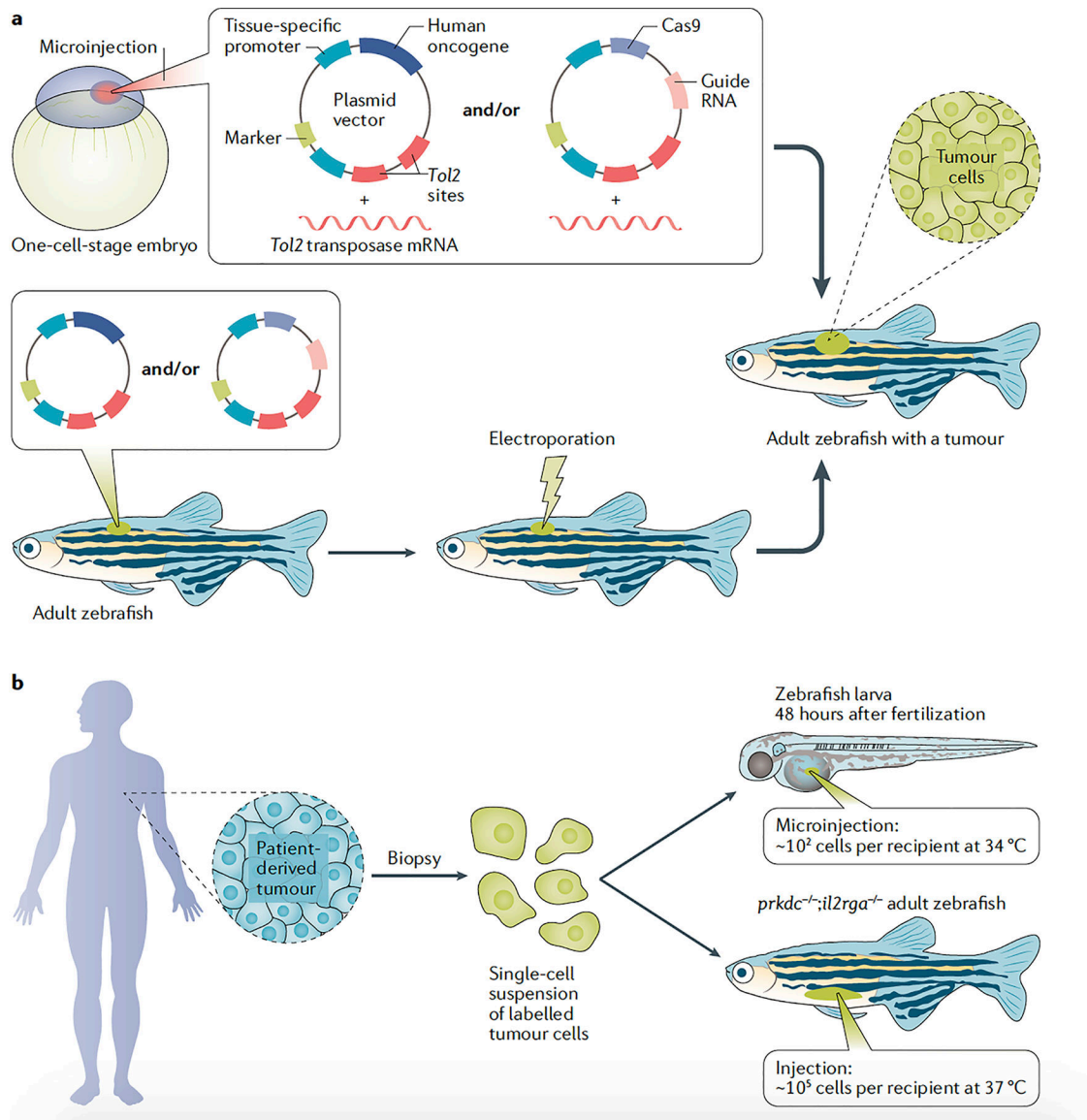
96. Liu PH et al. An IRAK1-PIN1 signalling axis drives intrinsic tumour resistance to radiation therapy. *Nat. Cell Biol* 21, 203–213 (2019). [PubMed: 30664786]
97. Smith MP et al. Effect of SMURF2 targeting on susceptibility to MEK inhibitors in melanoma. *J. Natl Cancer Inst* 105, 33–46 (2013). [PubMed: 23250956]
98. Yoganantharajah P & Gibert Y The use of the zebrafish model to aid in drug discovery and target validation. *Curr. Top. Med. Chem* 17, 2041–2055 (2017). [PubMed: 28137236]
99. Sarmah S & Marrs JA Zebrafish as a vertebrate model system to evaluate effects of environmental toxicants on cardiac development and function. *Int. J. Mol. Sci* 17, 2123 (2016).
100. Chakraborty C, Sharma AR, Sharma G & Lee SS Zebrafish: a complete animal model to enumerate the nanoparticle toxicity. *J. Nanobiotech* 14, 65 (2016).
101. Kanungo J, Cuevas E, Ali SF & Paule MG Zebrafish model in drug safety assessment. *Curr. Pharm. Des* 20, 5416–5429 (2014). [PubMed: 24502596]
102. Chakravarthy S, Sadagopan S, Nair A & Sukumaran SK Zebrafish as an in vivo high-throughput model for genotoxicity. *Zebrafish* 11, 154–166 (2014). [PubMed: 24428353]
103. Peterson RT & Macrae CA Systematic approaches to toxicology in the zebrafish. *Annu. Rev. Pharmacol. Toxicol* 52, 433–453 (2012). [PubMed: 22017682]
104. Boumahdi S & de Sauvage FJ The great escape: tumour cell plasticity in resistance to targeted therapy. *Nat. Rev. Drug Discov* 19, 39–56 (2020). [PubMed: 31601994]
105. Yao Y et al. Patient-derived organoids predict chemoradiation responses of locally advanced rectal cancer. *Cell Stem Cell* 26, 17–26.e6 (2020). [PubMed: 31761724] This article reports the use of organoids to assess drug response in the neoadjuvant setting.
106. Fazio M & Zon LI Fishing for answers in precision cancer medicine. *Proc. Natl Acad. Sci. USA* 114, 10306–10308 (2017). [PubMed: 28916734]
107. Baeten JT & de Jong JLO Genetic models of leukemia in zebrafish. *Front. Cell Dev. Biol* 6, 115 (2018). [PubMed: 30294597]
108. Kurata M et al. Highly multiplexed genome engineering using CRISPR/Cas9 gRNA arrays. *PLoS one* 13, e0198714 (2018). [PubMed: 30222773]
109. Zhang Y et al. A gRNA-tRNA array for CRISPR-Cas9 based rapid multiplexed genome editing in *Saccharomyces cerevisiae*. *Nat. Commun* 10, 1053 (2019). [PubMed: 30837474]
110. Kuzu OF, Nguyen FD, Noory MA & Sharma A Current state of animal (mouse) modeling in melanoma research. *Cancer Growth Metastasis* 8 (Suppl. 1), 81–94 (2015). [PubMed: 26483610]
111. Xue W et al. CRISPR-mediated direct mutation of cancer genes in the mouse liver. *Nature* 514, 380–384 (2014). [PubMed: 25119044]
112. Sanchez-Rivera FJ et al. Rapid modelling of cooperating genetic events in cancer through somatic genome editing. *Nature* 516, 428–431 (2014). [PubMed: 25337879] This article reports mosaicism transgenesis in mouse cancer models.
113. National Research Council. Guide for the Care and Use of Laboratory Animals 8th edn (National Academies Press, 2011)
114. US National Library of Medicine. [ClinicalTrials.gov https://clinicaltrials.gov/ct2/show/NCT03668418](https://clinicaltrials.gov/ct2/show/NCT03668418) (2018).
115. Vlachogiannis G et al. Patient-derived organoids model treatment response of metastatic gastrointestinal cancers. *Science* 359, 920–926 (2018). [PubMed: 29472484]
116. Tiriach H et al. Organoid profiling identifies common responders to chemotherapy in pancreatic cancer. *Cancer Discov.* 8, 1112–1129 (2018). [PubMed: 29853643]
117. Ganesh K et al. A rectal cancer organoid platform to study individual responses to chemoradiation. *Nat. Med* 25, 1607–1614 (2019). [PubMed: 31591597]
118. Ben-David U, Beroukhi R & Golub TR Genomic evolution of cancer models: perils and opportunities. *Nat. Rev. Cancer* 19, 97–109 (2019). [PubMed: 30578414]
119. Wang K et al. Patient-derived xenotransplants can recapitulate the genetic driver landscape of acute leukemias. *Leukemia* 31, 151–158 (2017). [PubMed: 27363283]
120. Ben-David U et al. Patient-derived xenografts undergo mouse-specific tumor evolution. *Nat. Genet* 49, 1567–1575 (2017). [PubMed: 28991255] This article shows the evolution of mouse PDXs compared with donor patients.

121. Ooft SN et al. Patient-derived organoids can predict response to chemotherapy in metastatic colorectal cancer patients. *Sci. Transl Med* 11, eaay2574 (2019). [PubMed: 31597751]
122. Hirata E & Sahai E Tumor microenvironment and differential responses to therapy. *Cold Spring Harb. Perspect. Med* 10.1101/cshperspect.a026781 (2017).
123. Binnewies M et al. Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat. Med* 24, 541–550 (2018). [PubMed: 29686425]
124. Vargas R et al. Case study: patient-derived clear cell adenocarcinoma xenograft model longitudinally predicts treatment response. *NPJ Precis. Oncol* 2, 14 (2018). [PubMed: 30202792]
125. Nardella C, Lunardi A, Patnaik A, Cantley LC & Pandolfi PP The APL paradigm and the ‘co-clinical trial’ project. *Cancer Discov.* 1, 108–116 (2011). [PubMed: 22116793]



**Fig. 1 |. Timeline of key developments in zebrafish cancer avatars.**

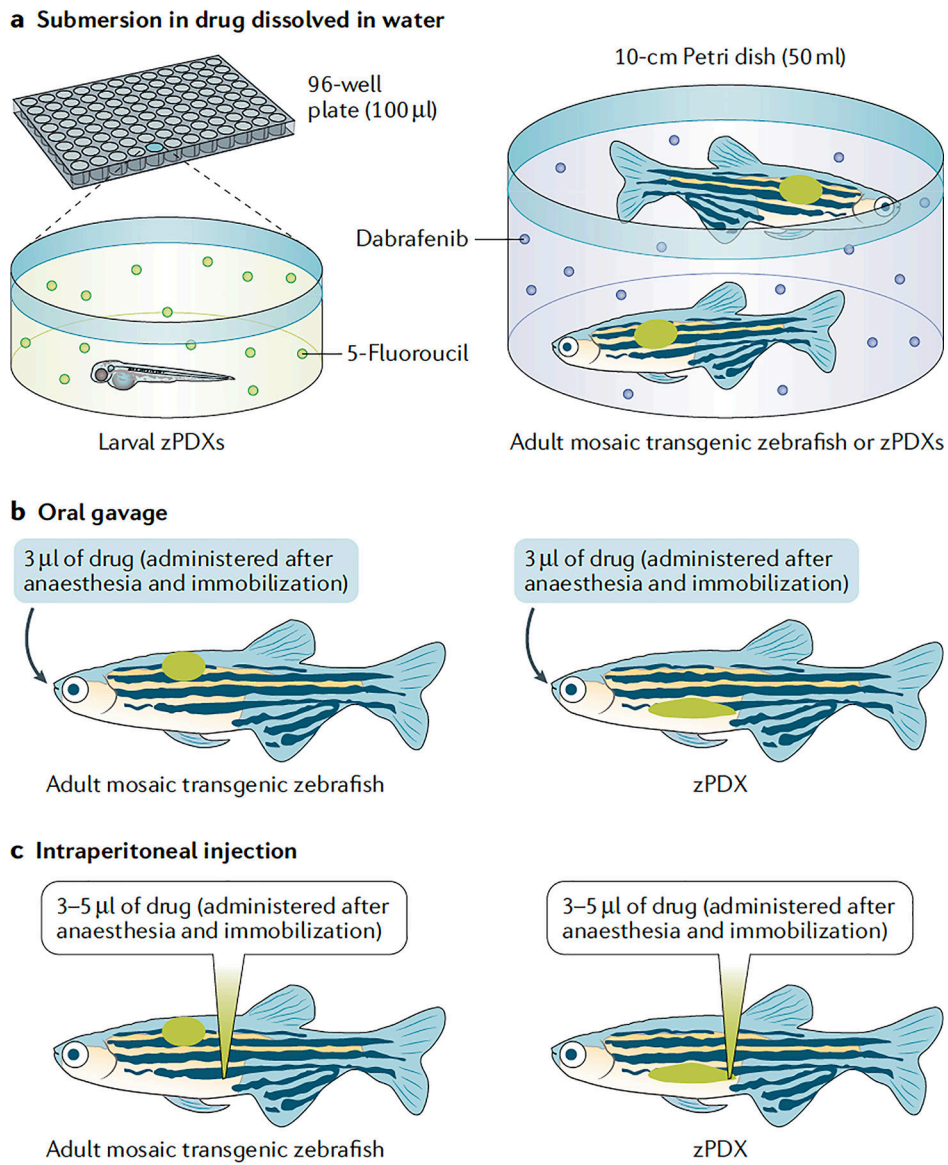
Timeline highlighting key milestones in the development of zebrafish cancer models towards personalized patient avatars (beige boxes). Salient milestones in clinical translation of cancer therapy response prediction using mouse, organoid and zebrafish patient avatars (purple boxes). PDX, patient-derived xenograft; zPDX, zebrafish patient-derived xenograft.



**Fig. 2 | Generation of zebrafish avatars.**

**a** | Mosaic transgenic zebrafish can be created using embryo microinjection or electroporation in adults. In embryos (top panel), *Tol2* mRNA is co-injected with plasmid DNA vector(s) containing *Tol2* sites, which are recognized by Tol2 protein and inserted in the host cell genome. For transgene electroporation in adult zebrafish (bottom panel), gene delivery is accomplished by co-injection of a DNA plasmid expressing *Tol2* with transgenesis vector(s) before electroporation. Between *Tol2* sites, transgenesis vectors typically include a (1) tissue-specific promoter driving a coding sequence, often a human oncogene<sup>12,23,34,36</sup>, or Cas9 for CRISPR knockout of tumour suppressors<sup>12,18</sup>, and (2) a transgenesis marker. The marker is often composed of a tissue-specific promoter and a fluorescent protein (for example, heart marker *cmhc2:EGFP*) or, in the case of melanoma, an *mitfa* minigene (promoter plus coding sequence to rescue the loss of *mitfa*), which allows melanocyte development itself to be used as a transgenesis marker in F0 zebrafish<sup>18,34</sup>. **b** |

Zebrafish patient-derived xenografts can be created by transplantation of cancer cells (which are usually labelled) in 48-hour post-fertilization larvae raised at 34 °C or genetically immunocompromised adult zebrafish (*prkdc<sup>-/-</sup>;il2rga<sup>-/-</sup>*) raised at 37 °C. Zebrafish embryos and adults are kept at 26–28.5 °C, while human cells grow at 37 °C. To support human cell engraftment, embryos are raised at 34 °C (ref.<sup>13</sup>), which, while below the normal physiological temperature for human cells, is the upper limit tolerated by zebrafish embryos and larvae. Adult fish can tolerate living at 37 °C for months if the temperature is slowly raised from 28.5 to 37 °C by about 0.5–1 °C per day<sup>14</sup>.



**Fig. 3 | Drug administration in zebrafish.**

**a** | Submersion in a water and drug solution is used to treat larval zebrafish patient-derived xenografts (zPDXs) (10–20 24-hour post-fertilization embryos per well in a 24-well plate or one embryo per 96-well plate) or adult avatars (two adults in a 10-cm Petri dish). Example compounds shown were used by Fior et al.<sup>13</sup> (5-fluorouracil treatment of larvae) and Ablain et al.<sup>12</sup> (treatment of adults with the BRAF inhibitor dabrafenib). **b** | Oral gavage can also be used to treat adult avatars (mosaic transgenic zebrafish or zPDXs). Zebrafish are anaesthetized and immobilized using MS-222 alone or in combination with isoflurane. Oral gavage using a syringe with flexible tubing allows ~3  $\mu$ l of drug solution to be dispensed (described by Dang et al.<sup>79</sup>). **c** | Intraperitoneal injection is another alternative to treat adult avatars (mosaic transgenic zebrafish or zPDXs). Zebrafish are anaesthetized and immobilized using MS-222 or MS-222 and isoflurane. Free-hand injections are performed



with 10- $\mu$ l capacity syringes to deliver up to 5  $\mu$ l of drug solution. Approximately 3–5  $\mu$ l is the tolerable range reported<sup>14,79</sup>.

Author Manuscript




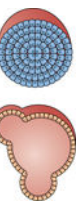

Author Manuscript

Author Manuscript

Author Manuscript

Table 1 |

Comparison of personalized cancer avatars

Cancer avatar type	Assay duration	Cost <sup>a</sup>	Logistic footprint <sup>a</sup>	Number of cells per recipient or condition	Drug screening throughput <sup>b</sup>	Cancer cell intrinsic target conservation	Patient TME conservation	Pharmacokinetic and dose optimization
Mosaic transgenic zebrafish 	Weeks to months	Low	Medium	NA (requires only patient tumour DNA sequencing)	Medium	100% for transgenic drivers; variable for others	No	Yes
Zebrafish PDXs in larvae 	5–7 days	Low	Low	10 <sup>2</sup>	High	High	No	No
Zebrafish PDXs in adults <sup>c</sup> 	Weeks to Months	Low	Medium	10 <sup>5</sup>	Medium	High	Low or no	Yes
Human 3D organoids in vitro 	1–2 weeks	Low	Low	10 <sup>2</sup> –10 <sup>3</sup>	Very high	High	No	No
Mouse PDXs 	Weeks to months	High	High	10 <sup>5</sup> –10 <sup>6</sup>	Low	High	Low or no	Yes

NA , not applicable; PDX, patient-derived xenograft; TME, tumour microenvironment.

<sup>a</sup>While cost and husbandry rules differ significantly by country and across institutions, previously reported cost and housing rules in North America and some European countries suggest that husbandry physical footprint space requirements (12 adult zebrafish versus 1 adult mouse per ~1.2 l)<sup>11,3</sup> and cost per day (US cents per 3.5- l fish tank versus US dollars per mouse cage, approximately \$1.05 per mouse versus approximately \$0.01 per adult zebrafish)<sup>45</sup> are at least an order of magnitude lower for zebrafish than for mice.

<sup>b</sup>Drug screening throughput order of magnitude assuming a duration compatible with clinical decision-making (days to weeks): low (1–5 compounds), medium (10–50 compounds), high (~100 compounds), very high (~1000 compounds).

<sup>c</sup>The data here are based on the proof-of-concept study by Yan et al.<sup>14</sup>. Further studies will be needed in adult zebrafish PDXs to generalize engraftment rates, assay duration and cell numbers across tumour types.