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Zebrafish In Vivo Models of Cancer and Metastasis

Katy R. Astell and Dirk Sieger

The Centre for Discovery Brain Sciences, The University of Edinburgh, Edinburgh EH16 4SB, United Kingdom

Correspondence: katy.astell@ed.ac.uk; dirk.sieger@ed.ac.uk

Metastasis, the dispersal of cancer cells from a primary tumor to secondary sites within the body, is the leading cause of cancer-related death. Animal models have been an indispensable tool to investigate the complex interactions between the cancer cells and the tumor microenvironment during the metastatic cascade. The zebrafish (*Danio rerio*) has emerged as a powerful vertebrate model for studying metastatic events in vivo. The zebrafish has many attributes including ex-utero development, which facilitates embryonic manipulation, as well as optically transparent tissues, which enables in vivo imaging of fluorescently labeled cells in real time. Here, we summarize the techniques which have been used to study cancer biology and metastasis in the zebrafish model organism, including genetic manipulation and transgenesis, cell transplantation, live imaging, and high-throughput compound screening. Finally, we discuss studies using the zebrafish, which have complemented and benefited metastasis research.

The zebrafish (*Danio rerio*) has become one of the most important vertebrate model organisms in biomedical research. Since the initial studies into genetics and vertebrate development, the zebrafish model has developed into a powerful system for studying many human diseases, including cancer biology, and has been used for anticancer drug discovery. Herein, we will review how the zebrafish model has benefited cancer research with an emphasis on metastasis. We believe that a good representation of the field is provided, however, due to the focus on metastasis we were not able to include every publication on the zebrafish cancer model.

EMERGENCE OF THE ZEBRAFISH AS A VERTEBRATE MODEL ORGANISM

First described by Francis Hamilton in "an account of the fishes found in the river Ganges and its branches" (Hamilton 1822), the zebrafish was originally a popular species for aquarium hobbyists. The potential of the zebrafish as a vertebrate model organism was first recognized by George Streisinger in the late 1960s (Grunwald and Eisen 2002), who published the first paper describing the use of the zebrafish and gynogenetic methods to identify recessive phenotypes from maternal genomes (Streisinger et al. 1981). At the same time, the zebrafish was becoming

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popular for forward genetic studies of development (Grunwald et al. 1988; Felsenfeld et al. 1990; Hatta et al. 1991). This led to the "big screen," in the 1990s, in which the zebrafish was the first vertebrate to be used for a large-scale genetic screen to identify developmentally important genes. Christiane Nüsslein-Volhard, in Tübingen and Marc Fishman in Boston performed these screens, in parallel, using *N*-ethyl-*N*-nitrosourea (ENU) as a chemical mutagen (Driever et al. 1996; Haffter et al. 1996). Thousands of mutations that affected multiple developmental processes were identified. Further characterization found that many of the zebrafish phenotypes resembled human diseases due to alterations in orthologous genes in the two species. The potential of the zebrafish as a model organism for human disease was now recognized and as a result, the National Institutes of Health established the Trans-NIH Zebrafish Coordinating Committee in 1997 (Grunwald and Eisen 2002). The original zebrafish genetic map (Postlethwait et al. 1994) was expanded (Shimoda et al. 1999) and several zebrafish models of the human disease quickly followed (Brownlie et al. 1998; Wang et al. 1998; Childs et al. 2000). Further development of the model facilitated forward and reverse genetic approaches, genetic manipulation, and transgenesis (Nasevicius and Ekker 2000; Talbot and Hopkins 2000). In the early 2000s, The Sanger Institute embarked upon the Zebrafish Genome Sequencing Project (Howe et al. 2013). In addition, the Zebrafish Information Network (https://zfin.org/) was established (Sprague et al. 2001) as well as the Zebrafish International Resource Center, a central repository for wild-type, mutant, and transgenic zebrafish lines as well as information about zebrafish research. Today, a PubMed search for "zebrafish" will return over 35,000 items. New tools continue to be developed for the zebrafish, including advancements in genome editing using the CRISPR/Cas9 system (Hruscha et al. 2013; Hwang et al. 2013; Stella and Montoya 2016) and automated screening platforms, such as the vertebrate automated screening technology (VAST) (Pardo-Martin et al. 2010; Chang et al. 2012; Early et al. 2018). This will ensure that the zebrafish remains an important vertebrate model organism today and in the future.

ZEBRAFISH DEVELOPMENT AND THE FOUR LIFE STAGES

Zebrafish are small in size and are easy to breed and maintain in large numbers at low costs. Laboratory zebrafish are housed in purpose-built aquaculture systems, maintained at 28.5°C with a controllable light–dark cycle. Zebrafish are highly fecund and a gravid female can produce hundreds of eggs each week through natural spawning. Fertilization and embryo development both occur externally to the female. Embryonic development is rapid, within 24 h the single-celled zygote develops into a motile, transparent embryo with a classical vertebrate body plan (Kimmel et al. 1995). Maintained at 28.5°C, the majority of morphogenesis is complete by 3 d postfertilization (dpf) and the embryo will hatch from the protective chorion. This marks the transition from the embryonic to the larval stage. The larval zebrafish will continue to grow and between 5 and 6 dpf the digestive system and mouth become functional. The yolk sac which has sustained the animal throughout the embryonic and early larval stages is now rapidly depleted and is completely absorbed by 7 dpf (Kimmel et al. 1995). The zebrafish is considered juvenile once it has developed the majority of adult characteristics and an adult when it can produce viable gametes and reproduce (Kimmel et al. 1995; Parichy et al. 2009). Under optimal conditions, laboratory zebrafish will reach sexual maturity during the third month of their development.

THE ADVANTAGES OF USING THE ZEBRAFISH TO MODEL CANCER BIOLOGY

The zebrafish has several attributes that make it a suitable model for investigating human cancer biology and metastasis. Many of the factors involved in tumor progression are highly conserved between zebrafish and humans. The zebrafish genome contains 26,206 protein-coding genes. When compared to the human genome, 71.4% of human genes were found to have at

least one zebrafish orthologue, while 82% of human disease-related genes had at least one zebrafish orthologue (Howe et al. 2013). In addition, multiple epigenetic markers regulating gene expression are also conserved across vertebrates including the zebrafish and humans (Long et al. 2013). Although many of the cell cycle genes, tumor suppressors, and oncogenes are conserved, allowing these tumorigenic pathways to be studied and targeted in the fish, it should be noted that zebrafish do not express clear orthologues of several human genes known to be involved in cancer progression. These include leukemia inhibitory factor (LIF), oncostatin M (OSM), and breast cancer 1 early onset (BRCA1) (Howe et al. 2013). However, as the receptors for these proteins are present in the zebrafish genome, it is possible that zebrafish proteins with similar functions to LIF and OSM exist, but sequence diversity is too great to recognize them as orthologues. In addition, a genome duplication has occurred in the Teleost fish genome following the phylogenetic divergence of fish and mammals (Meyer and Schartl 1999). This has resulted in the zebrafish having two copies of many genes (known as ohnologues) of which mammals only have one copy.

Zebrafish are easy to genetically manipulate through gene knockdowns, knockouts, overexpression, and transgenesis allowing the creation and characterization of cancer models. Importantly, there are similarities between the histopathology of human and zebrafish tumors (Amatruda et al. 2002). The rapid ex-utero development of the fertilized egg allows direct visualization and manipulation throughout embryogenesis and larval development. The transparency of the animal at these stages in combination with the generation of transgenic fluorescent reporter lines allows cellular and subcellular in vivo imaging to be performed allowing the tumor and its microenvironment to be monitored in real time. This has been one of the greatest benefits of using zebrafish to study cancer biology.

There is temporal separation in the maturation of the innate and adaptive immune systems in the zebrafish. The innate immune system is functional at 2 dpf, however, the adaptive immune system matures at 28 dpf (Lam et al. 2004). The lack of adaptive immunity in the larval zebrafish makes it an ideal in vivo model for the injection of cancerous cells without risk of rejection. Cells can either be allografted from a donor zebrafish (Langenau et al. 2003) or xenografted from a mammalian source (Nicoli et al. 2007). Proliferation, invasion, and metastasis of the injected cells can be investigated, as well as the interactions between the donor cells and both the host cells and the extracellular environment. However, this may not fully reproduce the behavior of cancer in an immunocompetent host.

TECHNIQUES USED TO STUDY CANCER BIOLOGY AND METASTASIS IN THE ZEBRAFISH

Genetic Manipulation

An important method in zebrafish research is the ability to induce cancers by expressing genes, including human oncogenes, in specific cells at specific time points. The transient cell-specific expression of oncogenes can be achieved using Tol2 mediated integration (Kawakami et al. 2004) which allows the early stages of cancer progression to be investigated. We have used this technique to express the human AKT1 oncogene in the neural cells of larval zebrafish. Based on this method, we showed that AKT1 activation in preneoplastic cells attracted macrophages via Sdf1b–Cxcr4b signaling, which in turn promoted oncogenic cell proliferation (Fig. 1A; Chia et al. 2018).

Spatial and temporal control of oncogene expression is often required to prevent oncogene toxicity or untimely death due to tumor burden. This has been achieved in the zebrafish by using the Gal4-UAS expression system (Scheer and Campos-Ortega 1999; Santoriello et al. 2010) the chemically inducible Tet-On system (Gossen and Bujard 1992; Gossen et al. 1995; Li et al. 2013) and LexPR:LexOP system (Emelyanov and Parinov 2008; Nguyen et al. 2012), as well as heat shock induction (Bajoghli et al. 2004; Leacock et al. 2012). Oncogenesis has also

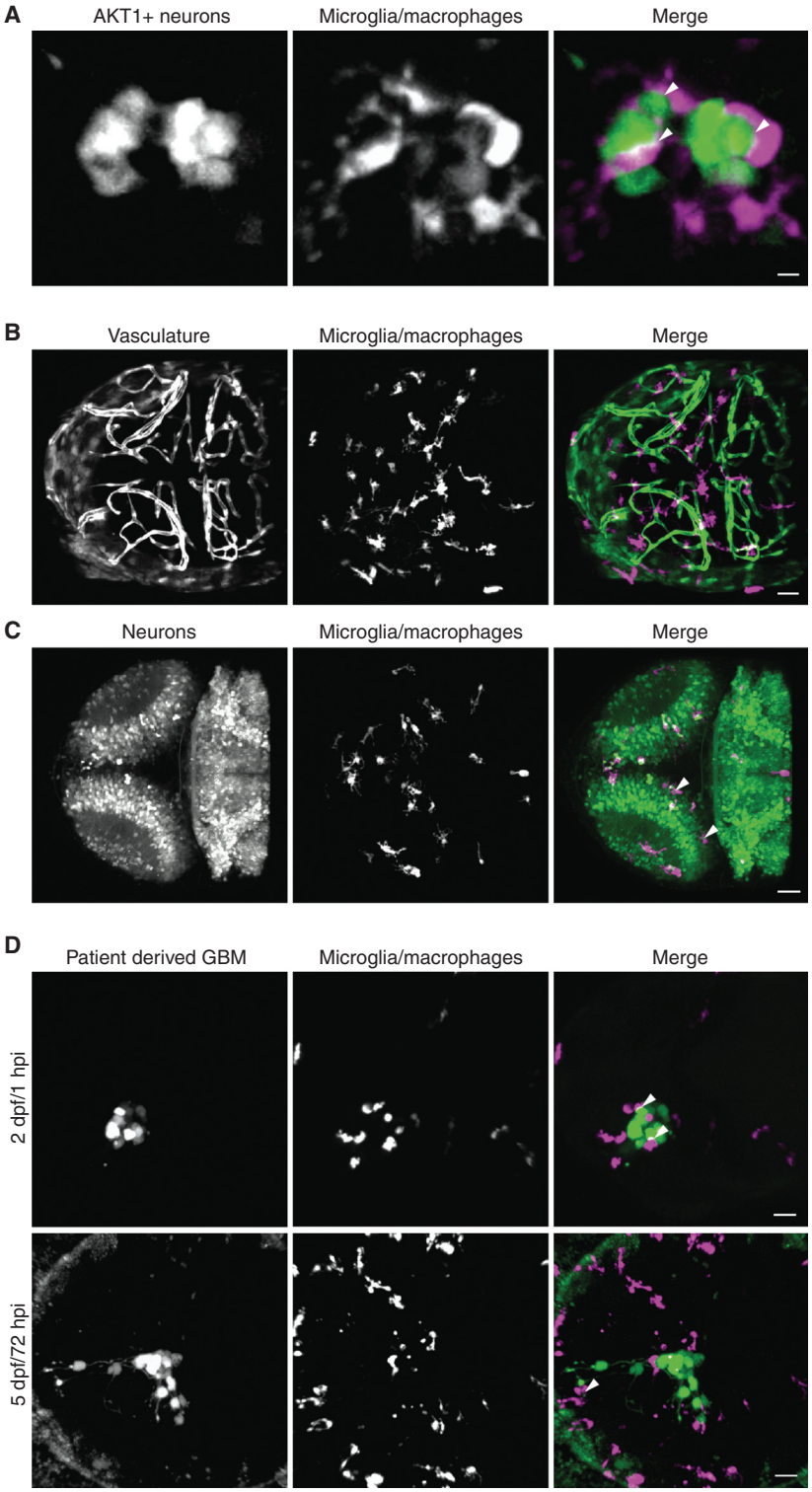

Figure 1. Confocal fluorescent microscopy enables an analysis of cell interactions, in vivo and in real time. (*A*) Oncogenesis was achieved in zebrafish neurons (green) by misexpression of a constitutively active form of human AKT1. Direct cellular interactions (white arrowheads) between the oncogenic neurons and microglia/macrophages (magenta) were visualized in vivo in real time. (*Legend continues on following page.*)

been achieved by transgene electroporation in adult zebrafish (TEAZ). Using this technique, oncogenes can be spatiotemporally expressed directly in adult somatic tissue, modeling tumor initiation and progression in a fully immunocompetent adult zebrafish (Callahan et al. 2018). Importantly, many of the zebrafish cancers generated by oncogene misexpression phenotypically copy the human equivalents (Santoriello et al. 2010). Furthermore, techniques are in place to isolate cells from zebrafish larvae followed by purification via flow cytometry to perform downstream applications including transcriptomic profiling (Mazzolini et al. 2018).

Cancers can also be induced by targeted gene knockdown. Effective gene knockdown can be achieved by microinjection of the one-cell stage embryo (Fig. 2). Morpholino antisense oligonucleotides (Nasevicius and Ekker 2000) can rapidly reveal the function of a gene, however, the phenotypes of morphants do not always correlate with those of stable mutants, implicating morpholino off-target effects (Kok et al. 2015). Synthetic transcription activator-like effector nucleases (TALENs) (Huang et al. 2011b; Sander et al. 2011) and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system (Hruscha et al. 2013; Hwang et al. 2013; Stella and Montoya 2016) can be used to generate inheritable frameshift knockout mutations. However, TALENs have proved difficult to design, synthesize, and validate, preventing widespread adoption of the technique (Gupta and Musunuru 2014). The CRISPR/Cas9 system is robust and can be used to knockout tumor repressor genes to modify the tumor microenvironment. It can also be used to knock-in genes, such as human cytokines or receptors which may be useful for future transplantation studies. However, knockout mutants must be validated to ensure that alternative start sites or splice sites cannot still generate a functional protein or that hypomorphic alleles are generated with reduced gene function. There is also evidence that complete gene knockout can be rescued by genetic compensation from other related genes, which can prevent the appearance of mutant phenotypes (Rossi et al. 2015).

Zebrafish Transgenics and Pigment Mutants

By combining transgenic lines and transparent zebrafish strains, the zebrafish becomes a powerful system for observing interactions between cancer cells and the surrounding environment. Live confocal fluorescent imaging enables an analysis of cell interactions in vivo and in real time. Hundreds of fluorescent reporter transgenic zebrafish lines have been established, labeling multiple cell types including blood vessels (Fig. 1B; Lawson and Weinstein 2002), immune

Figure 1. (*Continued*) The image is a maximum intensity projection, scale bar is 5 µm. (*B*,*C*) By combining fluorescent reporter transgenic lines and transparent zebrafish strains, the zebrafish becomes a powerful system for observing cellular interactions and tissue structures. (*B*) The *Tg*(*fli1:GFP:mpeg1:mCherry*) double transgenic zebrafish allows the vasculature (green) and microglia/macrophages (magenta) to be visualized in the zebrafish brain. (*C*) *Tg*(*nbt:dsRed:mpeg1:EGFP*) double transgenic zebrafish allow visualization of neurons (green) and microglia/macrophages (magenta) in the zebrafish brain. White arrowheads indicate apoptotic neurons that have been phagocytozed by the microglia/macrophages and are being digested within phagosomes. Images are maximum intensity projections showing the dorsal view of representative 3 d postfertilization (dpf) zebrafish embryo brains. Scale bars are 30 µm. (*D*) The invasiveness and cellular interactions of xenografted patient-derived tumor cells within the zebrafish brain can be investigated over time. Human, patient derived, glioblastoma multiforme cells (GBM; green) were transplanted into the left optic tectum of 2 dpf *Tg*(*mpeg1:mCherry*) zebrafish larvae. The larvae were imaged at 1 h postinjection (hpi) and at 72 hpi. Direct cellular interactions between the human tumor cells and the zebrafish microglia/macrophages (magenta) were observed at both time points (white arrowheads). The human cancer cells not only survived and proliferated within the zebrafish brain, but also exhibited an invasive phenotype, becoming more dispersed within the brain and extending long cellular processes into the surrounding brain tissue. Images are maximum intensity projections showing the dorsal view of a representative 2 dpf and a 5 dpf zebrafish embryo brain. Scale bars are 30 µm.

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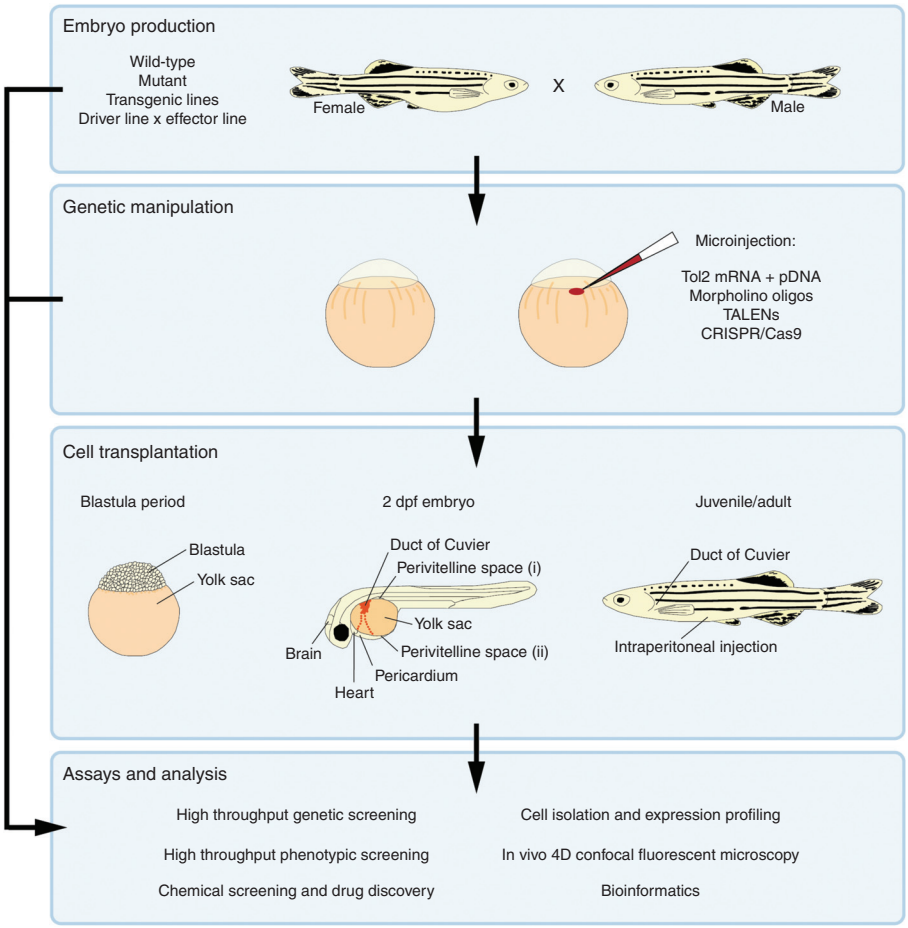

Figure 2. An overview of the common zebrafish techniques used to study cancer biology and metastasis. Zebrafish embryos are obtained through natural spawning of wild-type, mutant or transgenic fish. Oncogene expression can be achieved by crossing a cell-specific driver line with an effector line carrying the oncogene of interest. Embryos can be genetically manipulated by microinjection of reagents into the single-cell embryo. Tol2 mediated integration can be used to express oncogenes in specific cell types, morpholino antisense oligonucleotides can be used to knockdown gene expression and TALENs or the CRISPR/Cas9 system can be used to knockout target genes. Transplantation of cancer cells into zebrafish has been performed at several developmental stages. Fluorescent labeling allows continuous visualization of the transplanted cells, tumor progression and metastasis. The most common zebrafish developmental stages used for transplantation as well as the most frequently used injection sites are shown. These techniques can be combined to produce zebrafish embryos and larvae for further analysis including high-throughput genetic and phenotypic screening, chemical screening and drug discovery, gene expression profiling and in vivo 4D confocal fluorescent microscopy.

cells (Fig. 1B,C; Renshaw et al. 2006; Ellett et al. 2011), and neurons (Fig. 1C; Peri and Nüsslein-Volhard 2008). There are also transgenic zebrafish that can be used to drive oncogene expression. For example, the *Tg*(*5XUAS:eGFP-HRASV12*)$_{io006}$ effector line (Santoriello et al. 2010) can be used in combination with a Gal4 driver line to express a constitutively active form of HRas in a specific cell type.

The zebrafish embryo is transparent until 2 dpf, however, this transparency can be prolonged for a further 12 d through the use of melanin synthesis inhibitors (Karlsson et al. 2001). In addition, there are several pigment

mutants such as nacre (Lister et al. 1999) and crystal (Antinucci and Hindges 2016). The casper pigment mutant (White et al. 2008) has proved popular for cell transplantation studies as it lacks both iridophores and melanocytes and maintains transparency as an adult fish. Using immunosuppressed adult casper zebrafish and an allografting technique White et al. (2008) were able to assess engraftment and proliferation of either pigmented or fluorescent-labeled melanoma cells. Metastatic melanoma cell invasion from the primary injection site into the host tissues could be followed by repeatedly imaging the same live animal without the need to sacrifice the animal.

Allograft Cancer Cell Transplantation

An allograft is the transfer of cells, tissues or organs from one animal to another of the same species. Transplantation into the embryonic or larval zebrafish prevents transplant rejection and the transparency of the animal at this age allows cancer cells to be visualized during engraftment, proliferation, migration, neovascularization, and therapeutic treatments. Allogeneic cancer cell transplantation into adult zebrafish requires immunosuppression either by chemical ablation using dexamethasone (Langenau et al. 2004; Stoletov et al. 2007) or a sublethal dose of γ-irradiation (Traver et al. 2004). However, both techniques may be hindered by toxicity and incompetent ablation of the immune cells (Smith et al. 2010), while the lack of a complete immune system prevents cancer cell–immune cell interactions from being studied. In addition, recovery of the immune system postirradiation will cause rejection of the transplanted cells and prevents long-term investigations.

To avoid the issues related to immunosuppression, the clonal (syngeneic) zebrafish model (Streisinger et al. 1981) can be used to perform tumor transplantations (Mizgirev and Revskoy 2010). Syngeneic zebrafish have fully competent, matched immune systems. Therefore, cancerous cells from a syngeneic donor can be transferred into a sibling recipient without rejection (Mizgireuv and Revskoy 2006). This removes the need for immunosuppression, allowing long-term tumor progression to be studied in the presence of a fully functional immune system and tumor microenvironment with little variance in the genetic background. Immune compromised mutant zebrafish have also been used for transplantation studies. The rag2^{E450fs} mutant zebrafish has no mature T cells and a variable reduction in functional B cells (Tang et al. 2014). Cells and tissues from unrelated individuals can be transplanted into these fish and malignant cells engraft without the need for immunosuppression or major histocompatibility complex matching (Tenente et al. 2014).

Xenograft Cancer Cell Transplantation

A xenograft is the transfer of cells, tissues or organs from an animal of one species to an animal of a different species. Orthotopic xenografts involve transplanting tumor cells into the same organ type from which the cancer originated. Heterotopic xenografts involve transplanting tumor cells into a different tissue from which the cells originated. The zebrafish larva has proved to be an excellent system for human cancer xenograft studies. Fluorescent labeling allows continuous visualization of the transplanted cells and tumor progression (Ignatius and Langenau 2011). Due to the immature adaptive immune system, the human cancer cells survive within the zebrafish larvae, invade surrounding tissue and metastasize. Importantly, transplanted human cells can communicate with zebrafish cells due to the conservation between human and zebrafish signaling pathways (Tulotta et al. 2016). Furthermore, these pathways can be genetically or chemically targeted. The first zebrafish xenograft involved transplanting human melanoma cells into the zebrafish blastula (Lee et al. 2005), however, cancer cells are more often injected into the zebrafish larva at 2–4 dpf when the body plan has become established. Several injection sites are commonly utilized in the larval xenograft model including the yolk sac, the perivitelline space (the area between the outer membrane of the yolk sac and the skin), the duct of Cuvier (connecting the heart to the trunk vasculature), the pericardium, the heart and the brain (Fig. 2).

Many xenograft studies have used commercially available human (Ren et al. 2017) and murine (Zhao et al. 2011) cancer cell lines. This can be advantageous as it reduces variability and the cells can be manipulated in vitro before transplantation. However, due to the selection pressures of long-term maintenance in tissue culture, established cell lines may no longer resemble primary cancers. Therefore, xenografting studies have also been performed with primary patient-derived cells (Marques et al. 2009; Mercatali et al. 2016; Gaudenzi et al. 2017). As primary patient cells are difficult to obtain and expensive to maintain, the larval xenograft model is again beneficial as only small cell numbers are required for each injection (Fig. 1D). Patient-derived cells from a range of different cancers have also been xenografted into adult immunocompromised zebrafish. The *prkdc*$^{D3612fs/D3612fs}$, *Il2rga*$^{Y91fs/+}$, *casper* mutant lacks T, B, and NK cells and the engrafted cancer cells were shown to have similar growth kinetics and histopathological features to those grown in immunodeficient NSG mice (Yan et al. 2019). Various cell-labeling methods were used to follow cell growth, migration and responses to chemotherapeutics at single-cell resolution in vivo and provided proof of principle that this model could be used to develop patient-specific therapeutic approaches in the future.

Human cancer cells have also been transplanted into juvenile zebrafish to study microtumor formation, cell invasion, and angiogenesis (Stoletov et al. 2007). All tissues are fully developed at this stage, therefore, xenografting cells at this time may be more representative of tumor progression in an adult system without interference from developmental signaling in the embryo or larva. However, immunosuppression is still required and microscopy is more challenging at this age as the tissues are opaque. As well as confocal microscopy, several additional noninvasive imaging techniques can be used to identify cancer cells in juvenile and adult zebrafish in vivo. These include ultrasound biomicroscopy (Goessling et al. 2007), 2-photon microscopy and the luciferase based IVIS spectrum in vivo imaging system.

High-Throughput Chemical Genetic Screening

Chemical genetics involves using small molecules to alter biological pathways. The zebrafish is an excellent in vivo animal model for large-scale chemical screening. High fecundity, small size, and ease of handling all facilitate high-throughput screening. The most common format has been the 96-well plate, which allows small volumes of potentially expensive compounds to be tested. The aqueous environment allows the solubilization of compounds allowing easy drug delivery and pathway targeting in a whole organism. The rapid ex-utero development, transparency of the embryo and availability of transgenic lines allows for visualization throughout development and the detection of desired phenotypes within a short time frame. Physiologically relevant events can be evaluated simultaneously with drug efficacy and toxicity within an individual animal.

The first high-throughput forward genetic chemical screen, using wild-type zebrafish, was performed to identify novel compounds that regulate developmental pathways and embryogenesis (Peterson et al. 2000). Since this initial study, in vivo chemical screens utilizing zebrafish have been used to identify novel cancer therapeutic compounds (Wang et al. 2010; Ridges et al. 2012; Astin et al. 2014). Transgenic zebrafish allow phenotypes to be detected by fluorescent microscopy. The *Tg*(*flk1:EGFP*) transgenic line, with EGFP labeled vasculature has been used to identify compounds that inhibit angiogenesis (Wang et al. 2010). *Tg*(*lyve1:egfp*)^{nz150}, which has EGFP labeled lymphatic vessels, has been used to identify compounds that inhibit lymphatic vessel growth (Astin et al. 2014) and the T-cell reporter line *Tg*(*lck:EGFP*) has been used to identify compounds with selective activity against leukemia (Ridges et al. 2012). Automation has increased the efficiency of high-throughput compound screening. Embryos can be automatically sorted and dispensed into multiwell plates (Pulak 2006; Veneman et al. 2013), automated robotic drug dosing and microinjection reduces variability (Wang et al. 2007; Huang et al. 2011a) and automated

high-resolution in vivo imaging is achievable using the VAST BioImager (Pardo-Martin et al. 2010; Chang et al. 2012; Early et al. 2018).

ZEBRAFISH STUDIES OF THE METASTATIC PROCESS

The first use of zebrafish in cancer research was reported in 1965. In this study, zebrafish were exposed to carcinogens and were found to develop neoplasms (Stanton 1965). Since this initial publication, the zebrafish has become a powerful vertebrate system for modeling human cancers (Stoletov and Klemke 2008) and is an excellent platform to study all stages of metastasis.

Metastasis is a multistep process that involves the migration of tumor cells away from the primary tumor site, intravasation into the circulatory or lymphatic system, survival in the vasculature, extravasation, and colonization at a secondary site in a foreign environment.

Angiogenesis and Local Invasion from the Primary Tumor

Angiogenesis is important for tumor growth and new vessels may provide a route for cell dissemination and metastasis from the primary tumor. The predictable patterning of the vasculature, optical clarity, and availability of transgenic lines with fluorescently labeled vasculature made the zebrafish an attractive model for studying tumor–vasculature interactions in real time and led to the development of the zebrafish/tumor xenograft angiogenesis assay (Nicoli and Presta 2007). Multiple cancer cell types, including metastatic melanoma, breast adenocarcinoma, and pancreatic cancer have been shown to stimulate angiogenesis when xenografted into the zebrafish (Haldi et al. 2006; Stoletov et al. 2007; Nicoli et al. 2007). Mammalian tumor cells expressing angiogenic vascular endothelial growth factor (VEGF) can rapidly induce microvascularization when xenografted close to the subintestinal vasculature of developing zebrafish embryos (Nicoli et al. 2007), indicating that there is functional signaling between the mammalian and zebrafish cells. In addition to growth factors, modeling tumor-induced angiogenesis in the zebrafish has successfully identified other molecular factors that regulate tumor cell-induced neovascularization in vivo. For example, angiogenesis was significantly reduced in a pancreatic cancer cell xenograft assay following siRNA silencing of either LIMK1 or LIMK2 in the cancer cells (Vlecken and Bagowski 2009).

As a tumor mass grows it must recruit a blood supply to prevent tissue ischemia. However, hypoxia can still occur as tumor-induced vasculature is often disorganized and leaky. It has been suggested that leaky vessels and hypoxia may promote metastasis of tumor cells. To further investigate how hypoxia drives metastasis, the Cao research group used a zebrafish hypoxia model to show that VEGF secreted from tumor cells promoted induction of pathological angiogenesis which increased tumor cell invasion, dissemination, and metastasis under hypoxic conditions (Lee et al. 2009; Rouhi et al. 2010). This was the first animal model that combined the study of tumor hypoxia, angiogenesis, and cancer cell metastasis at the single-cell level.

Macrophages in the tumor microenvironment are also involved in tumor-induced angiogenesis. He et al. reported a macrophage-dependent neovascularization when tumor cells were injected into the duct of Cuvier of 2 dpf *Tg*(*Fli1:GFP*) zebrafish, which have fluorescent vasculature (Lawson and Weinstein 2002). This was the first example of de novo vascularization in a zebrafish xenograft system which was essential for the proliferation of both the tumor cells and the stromal cells during tumor progression (He et al. 2012). In addition, it has been shown that macrophage infiltration into glioblastoma xenografts is enhanced by TGF-β1 expression and the JNK pathway (Yang et al. 2013). Britto et al. (2018) found that the angiogenic response to VEGFA secreting xenografted tumor cells can be enhanced by direct interactions between non-inflammatory macrophages and the growth tips of the developing blood vessels. Microglia, the resident macrophages of the central nervous system have also been shown to directly interact with human glioblastoma cells promoting the survival and invasiveness of the cancer cells

when xenotransplanted into the zebrafish midbrain (Hamilton et al. 2016).

Cancer cells can associate and actively migrate along the outer surface of blood vessels, as has been observed for human glioblastoma when xenotransplanted into the hindbrain ventricle of 2 dpf zebrafish embryos (Gamble et al. 2018). Therefore, angiogenesis may promote cancer cell dispersal from the original cell mass by providing a physical structure along which the cells can travel. In addition, an increased migratory invasive phenotype may aid the initial stages of metastasis. While exploring tumor cell–endothelial cell interactions, Stoletov et al. (2007) found that cancer cells expressing the metastatic gene RhoC were found to disseminate further from the site of xenotransplantation. This may give cancer cells a metastatic advantage by increasing the probability that they will come into contact with the vasculature and undergo intravasation.

Cooperative invasion is a mechanism by which heterogeneous tumor cell populations communicate reciprocally and cooperate to migrate collectively from the initial cell mass. Chapman et al. (2014) found that coinjecting highly invasive melanoma cells, that deposit extracellular matrix and exhibit protease activity, along with less invasive melanoma cells increased the invasive potential of the less invasive cells and promoted melanoma progression.

Immune cells have been shown to be involved in cancer cell dissemination. A study using the zebrafish xenograft model to investigate the interactions between tumor cells and macrophages in association with the vasculature revealed a novel mechanism of intravasation involving tumor-associated macrophages (TAMs) (Wang et al. 2015). A mixture of tumor cells and macrophages were injected into the perivitelline space of 2 dpf *Tg*(*Fli1:EGFP*) larvae to partially recapitulate the tumor microenvironment. The findings revealed that IL6- or TNFα-stimulated noninflammatory TAMs enhanced metastasis of the tumor cells at the stage of intravasation and dissemination. Metastasis was dependent upon direct cancer cell–TAM interactions, with the majority of the disseminated tumor cells associated with TAMs in vivo (Wang et al. 2015). This study revealed a novel macrophage-dependent mechanism of metastasis and supported data from mouse tumor models that had previously suggested that TAMs facilitate tumor cell intravasation (Wyckoff et al. 2007; Pollard 2008). In addition, the model provides a functional platform that could be used to test metastatic potential of patient tumor cells and screen for new therapeutic agents.

Intravasation and Survival in the Circulation

The zebrafish xenograft model has been highly valuable when investigating intravasation. To explore tumor cell–endothelial cell interactions, Stoletov et al. (2007) transplanted fluorescent human cancer cells into the peritoneal cavity of 1-mo old immunosuppressed zebrafish and observed the cellular behaviors by high-resolution imaging. RhoC was found to work cooperatively with VEGF to promote intravasation. The injected cells formed microtumors in the zebrafish and VEGF secretion from these cells was found to promote localized angiogenesis, vascular remodeling, and permeablization due to disruptions in the endothelial cell layer. The metastatic gene RhoC regulates the actin–myosin cytoskeleton. Cancer cells expressing RhoC exhibited amoeboid cell migration, promoting dispersal, as well as the formation of long membrane protrusions and blebs. These long cell protrusions were seen to extend through the VEGF induced openings in the vasculature and allowed the tumor cells to enter the vessels.

In a study to determine the mechanisms by which T-lymphoblastic lymphoma (T-LBL) progresses to T-cell acute lymphoblastic leukemia (T-ALL), Feng et al. (2010) studied allografts in *Tg*(*fli:egfp*); *casper* zebrafish and found that overexpression of b-cell lymphoma 2 (bcl2) in combination with c-myc expression prevented MYC-induced apoptosis and increased malignant thymocyte transformation. However, these leukemia cells were unable to transit into the vasculature due to increased adhesion caused by elevated levels of Sphingosine-1-phosphate receptor 1 (S1P1) and Intercellular Adhesion Molecule 1 (ICAM1). This study revealed that reduced intercellular adhesion is re-

quired for intravasation and dissemination during the transition from T-LBL to T-ALL.

To investigate the behavior of tumor cells within the circulation, the cells can be injected directly into the duct of Cuvier in the developing zebrafish. Frequent time-lapse confocal microscopy revealed human melanoma cells interacting with zebrafish endothelial cells within the vasculature. Melanoma cells were seen to alternate between rolling along the inside surface of the vessels and being stationary (Hill et al. 2018). This assay can be used to model how tumor cells circulate and disseminate in vivo and how the cells survive the hostile environment of the circulatory system.

Tumor Cell Homing and Extravasation

Metastasis of several hematological cancers, as well as solid tumors, indicates a preference for homing to specific predetermined distant tissues. The bone marrow is a common site for secondary tumor growth during metastasis (Shiozawa et al. 2015). A zebrafish model was established in which the homing of multiple myeloma cells to the caudal hematopoietic tissue (CHT) was investigated (Sacco et al. 2012). The zebrafish CHT is known to be an intermediate site of hematopoiesis and leukocyte differentiation (Chen and Zon 2009) and is equivalent to the mammalian fetal liver (Murayama et al. 2006). The CHT is thought to represent a bone marrow-like hematopoietic stem cell niche which may be involved in the homing of tumor cells to the bone marrow (Shiozawa et al. 2015). Multiple myeloma cells rapidly entered the peripheral circulation and migrated to the CHT after intracardiac injection into 2 dpf *casper* embryos. Transcriptomic analyses of the multiple myeloma cells that had localized to the CHT revealed enrichment for genes known to regulate IL6 signaling, angiogenesis, and cell adhesion. Furthermore, silencing the known bone marrow homing factors CXCR4 (Alsayed et al. 2007), VLA4 (Sanz-Rodríguez et al. 1999; Ngo et al. 2008), and FAK (Park et al. 2013) in the multiple myeloid cells reduced localization to the CHT. Thus, this zebrafish system complements the murine models, allowing small numbers of valuable patient-derived cancer cells that metastazise to the bone marrow to be studied and used for chemotherapeutic screening. It also provides further evidence that human cells can communicate with the host zebrafish cells. Cancer cell homing and colonizing of the CHT has also been reported in additional xenograft models (Chen et al. 2017).

To investigate the behavior of cancer cells extravasating from the circulation Stoletov et al. (2010) combined xenografting with real-time high-resolution imaging. Human cancer cells were injected directly into the circulation through the pericardium. The cancer cells were found to become lodged in the small vessels (5–8 μm) of the zebrafish head and tail. Extravasation from the vessels was an active process, dependent on the metastatic potential of the cells, and did not cause vasculature damage or leakage. Circulating tumor cells arrested at sites where the nuclei of the endothelial cells protruded into the vessel, suggesting that the arrest of circulating cells was due to size restriction and not due to adhesive mechanisms. Arrested cells were able to migrate along the inside surface of the vessel and could generate enough force to migrate against the blood flow. They also had the ability to alter their shape and move through vessel branching points by adjusting the actin–myosin contractile system and creating rounded membrane protrusions. A thickening of the vasculature cell wall surrounding the arrested tumor cells was observed. This was caused by increased movement and clustering of the endothelial cells and was accompanied by remodeling of the endothelial cell junctions. The pro-metastatic genes Twist, VEGFA and integrin β1 promoted migration, remodeling of the vasculature and extravasation of the tumor cells (Stoletov et al. 2010).

Further work by Kanada et al. (2014) detected two distinct mechanisms of extravasation in the zebrafish. RFP-labeled Hela cells were injected into the circulation of 2 dpf *Tg*(*flk1:EGFP*) embryos and the process of extravasation was observed by long-time fluorescent time-lapse imaging. As previously observed (Stoletov et al. 2010), some of the Hela cells arrested in the vasculature and then underwent VEGF-depen-

dent extravasation by actively penetrating the vessel walls. The second mechanism of extravasation occurred independently of VEGF signaling and involved stationary clusters of noninvasive Hela cells being progressively covered by a layer of endothelial cells. This continued until the Hela cells were excluded from the vessel, at which point the original endothelial cell layer disappeared and the Hela cells dispersed into the surrounding tissues (Kanada et al. 2014).

As well as the signaling between tumor cells and epithelial cells, there is evidence that immune cells can also have a role in promoting extravasation in vivo. Another study into tumor cell dispersal found that neutrophils can promote extravasation and invasion into secondary sites. He et al. (2012) injected fluorescent tumor cells into the duct of Cuvier of 2 dpf *Tg*(*Fli1: GFP*) transgenic zebrafish. Some of the injected cells were disseminated through the vasculature by the blood circulation. Extravasation of the dispersed tumor cells was observed at different locations throughout the larvae, however, only cells that had localized to the CHT survived and successfully invaded the surrounding tissue. This was found to be due to the high numbers of neutrophils in the CHT. Migration of neutrophils between the CHT and tail fin distorted the extracellular fibrillary collagen matrix forming a metastatic niche that promoted tumor cell invasion. As had been reported in mouse models and human patients treated with VEGFR inhibitors (Ebos et al. 2009; Loges et al. 2009; Pàez-Ribes et al. 2009), VEGFR inhibition was found to reduce tumor growth at the primary site, but promoted metastasis and cell invasion. This may be due to increased neutrophil migration generating pathways in the collagen, through which the tumor cells can migrate (He et al. 2012). Further study of the involvement of neutrophils during metastasis found that neutrophils in zebrafish larvae express high levels of cxcr4 (cxcr4b), which controlled the neutrophil number, adhesion, and motility (Tulotta et al. 2019). Tumor cells xenografted into cxcr4b homozygote mutant zebrafish failed to form micrometastazes in the CHT. This was due to either neutrophil migratory impairment, resulting in reduced metastatic niche formation, or the absence of cxcr4b-dependent tumor cell homing. Once the human tumor cells had extravasated through the CHT and invaded the surrounding tissue, a cxcr4b-dependent migration of neutrophils out of the CHT was observed. These neutrophils were seen to slow down and interact with the metastasizing cancer cells. These studies suggest that targeting cxcr4 on both tumor cells and neutrophils could have a therapeutic benefit to constrain cancer progression (Tulotta et al. 2019).

Colonization at a Secondary Site

The final stage of metastasis involves colonization of a secondary site in a foreign environment. Therefore, a metastatic niche capable of sustaining tumor growth must be established. To investigate this process further Ignatius et al. used the $KRAS^{G12D}$-induced embryonal rhabdomyosarcoma (ERMS) zebrafish model of an aggressive pediatric muscle sarcoma (Langenau et al. 2007) to identify tumor-propagating cells (Ignatius et al. 2012). ERMS-propagating cells expressing *myf5* exhibited enhanced proliferative capacity but minimal migratory potential. These cells were unable to undergo intravasation and were often restricted to the primary tumor mass. As differentiation progressed, a subset of ERMS cells expressing myogenin became highly migratory. These cells lack tumor-propagating potential, but, can transverse the vasculature and seed secondary sites for tumor growth. These secondary sites were later colonized by the slower moving ERMS-propagating cells (Ignatius et al. 2012). This work suggests that nontumor propagating cells may have important roles in facilitating tumor spread as well as maintaining the primary tumor microenvironment, which could have therapeutic implications.

After metastatic dissemination, the microenvironment of the secondary site can produce factors to promote phenotypic switching and survival of the tumor cells at the new location. The White laboratory used a zebrafish melanoma model to observe changes in cell differentiation during metastatic engraftment (Kim et al. 2017). This study identified that after extravasation melanoma cells undergo melanocytic

differentiation and proliferation, regulated by the microenvironmental factor Edn3. CRISPR/Cas9 mediated knockdown of this developmental morphogen prolonged survival of zebrafish transplanted with melanoma cells. This study emphasizes the significant role that the microenvironment plays in metastatic success and disease progression (Kim et al. 2017).

CONCLUSION

The zebrafish has proved itself as a suitable and useful model vertebrate to study tumor biology and complements the in vitro and in vivo mammalian cancer models. Techniques such as tumor cell transplantation assays, genetic manipulation, high-throughput chemical screening and in vivo live imaging have all enhanced our knowledge of cancer progression and metastasis. The zebrafish has been invaluable for single-cell high-resolution imaging and observing direct tumor cell interactions with the microenvironment during the more elusive stages of metastasis. The model is continuously being improved and advances in automation as well as the use of patient-derived xenografting assays can be used to screen in vivo drug responses and the kinetics of combinational therapies. In turn, this could lead to rapid patient-specific preclinical screening of cancer cell responses to specific drug combinations and guide treatment plans to target and prevent metastasis.

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