Zebrafish xenograft models of cancer and metastasis for drug discovery

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Abstract

Introduction: Patients with metastatic cancer suffer the highest rate of cancer-related death, but existing animal models of metastasis have disadvantages that limit our ability to understand this process. The zebrafish is increasingly used for cancer modelling, particularly xenografting of human cancer cell lines, and drug discovery, and may provide novel scientific and therapeutic insights. However, this model system remains underexploited, and we aim to inform non-specialists in a balanced and realistic manner.

Areas covered: We discuss the advantages and disadvantages of the zebrafish xenograft model for the study of cancer, metastasis and drug discovery. We summarise previous work investigating the metastatic cascade, such as tumour-induced angiogenesis, intravasation, extravasation, dissemination and homing, invasion at secondary sites, assessing metastatic potential and evaluation of cancer stem cells in zebrafish. Treatment studies aiming to disrupt metastases are also reviewed.

Expert opinion: The practical advantages of zebrafish for basic biological study and drug discovery are indisputable. However, their ability to sufficiently reproduce and predict the behaviour of human cancer and metastasis remains unproven. For this to be resolved will require novel mechanisms to be discovered in zebrafish that are subsequently validated in humans, and for therapeutic interventions that modulate cancer favourably in zebrafish to successfully translate to human clinical studies. In the meantime, more work is required to establish the most informative methods with which to study human cancer biology in zebrafish.

Keywords: cancer, metastasis, xenotransplantation, zebrafish

1. Introduction

Metastatic disease is the leading cause of cancer-related mortality, with advanced disease remaining incurable [1]. Metastasis is a complex multistep process involving invasion and detachment from the primary tumour, intravasation into the blood or lymphatic vessels, extravasation into predetermined secondary tissues, engraftment in a new microenvironment and eventually tumour proliferation [2-4]. The interactions of cancer cells with their surrounding milieu, the microenvironment and the metastatic niche are promising targets to prevent or inhibit tumour spread. However, our increasing understanding of intra- and inter-tumour heterogeneity suggests that mechanisms of metastasis may be variable both between patients and between different cancer cell types even within the same tumour [5, 6]. There therefore remains much to understand about the fundamental mechanisms of metastasis, and the inability to observe and experimentally manipulate human cancers clearly requires the use of animal models.

Although a wide range of model systems have been applied to study cancer metastasis for many years [7] (including murine xenograft, chick chorioallantoic membrane and *in vitro* models[8]) none entirely recapitulates or allows observation of all steps and factors involved in the metastatic cascade. Although space does not allow us to rehearse the advantages and disadvantages of all these models, the current paucity of effective anti-metastatic treatments underlines the rationale for seeking to exploit other model systems. In this review, we will focus on the advantages and disadvantages of the zebrafish for the study of cancer and metastasis, and as an emerging model of *in vivo* drug discovery.

2. Advantages and disadvantages of zebrafish models

Discussion of the zebrafish as an experimental model often focuses on its practical advantages. It is unarguable that their small size, low cost, the ability to generate hundreds of embryos from a single mating, and the less onerous regulatory oversight of studies using early embryos (at least in some jurisdictions) contrast favourably with rodents and are appealing to researchers, their institutions, and funding agencies [9]. However, we here focus on the purely scientific advantages of the zebrafish, balanced with some important considerations that should be taken into account.

The prominence of the zebrafish as a model for developmental biology is due to the advantage that the fertilised egg develops into a free-living adult external to the mother (common to other fish and amphibians)[10, 11]. This allows direct observation of the entire process of embryogenesis without instrumentation, providing a significant advantage over chick and mammalian models of vertebrate development. Embryogenesis occurs rapidly; the single cell fertilised egg (conveniently visible to the naked eye within its protective chorion) to a motile larva with a recognisable vertebrate body plan, beating heart, and functioning neurological system by 1 dpf (days post fertilisation, the conventional staging nomenclature of zebrafish) [12]. Over the next 5 days, the embryo grows and develops happily in Petri dishes incubated around 28 degrees Celsius while consuming the yolk contained in the vegetal pole of the original egg (thus not requiring feeding, or maintenance other than changing the medium occasionally). Although the overall morphology changes little between 1 to 5 dpf the zebrafish embryo becomes increasingly pigmented after 2 dpf (developing the stripes from which it obtained its name) and the vasculature and other organs undergo extensive expansion and remodelling [12, 13].

This continual and rapid development during the early days of life, though useful for embryologists, is perhaps a disadvantage for cancer studies, in which a more stable baseline

might be preferable. The innate immune system is functional by at least 2 dpf, whereas adaptive immunity develops later at around 4-6 weeks post fertilisation [14-16], meaning that studies in early embryos may not always reproduce the behaviour of cancers in a fully immunocompetent host.

The *sine qua non* of zebrafish experimentation is their ability to provide unrivalled *in vivo* cellular and subcellular imaging without instrumentation. This is due to a combination of the physical characteristics of the embryo (small size and relative transparency) and the ability to generate tissue-specific transgenics expressing a range of fluorescent reporters, for example to image blood vessels [17, 18], neurons[19], blood [20, 21] or immune cells [22, 23]. The availability of the single-cell embryo for injection with genetic constructs greatly facilitates transgenesis, and technical advances such as Tol2-mediated integration have made creation of novel transgenics relatively straightforward[24], although as it requires raising of founders and subsequent outcrossing, it still takes several months to generate and identify a stable transgenic line [25, 26].

It is important to note however, that the ability to obtain excellent imaging only applies to embryos of earlier ages and often requires prevention of pigmentation (such as the use of mutant lines such as the *Nacre* or *Casper* mutants [27]. With advancing age, the embryo becomes larger and more opaque, such that it is difficult to image deep within the animal. Although lightsheet imaging, also known as selective plane illumination microscopy (SPIM)[28, 29] allows deeper tissue penetration and less phototoxicity, it still cannot penetrate tissue of the size of an adult zebrafish, and immobilisation and anaesthesia of older fish is challenging. There is thus a point during development where the zebrafish becomes little if any better for in vivo imaging than other organisms, at which point its small

size and aquatic milieu can even be disadvantageous. The exact timing of this point does however clearly depend on the imaging required and the technical demands of the imaging modality.

While the zebrafish's main strength lies in its capacity for superb *in vivo* imaging, it conversely suffers some disadvantages for examination of fixed tissue. Sectioning embryos or larvae is tricky due to their small size, and there are only limited numbers of well-validated antibodies against zebrafish proteins (although this number is steadily increasing) making detailed histological analysis challenging.

The zebrafish genome is often described as "well conserved" with around 70% of human genes represented in the zebrafish genome [30]. An added complexity in zebrafish is that many human genes have more than one zebrafish orthologue (properly termed an *ohnologue*) thanks to an additional genome duplication experienced by the teleost ancestral line after its divergence from the mammalian. Even human genes with only one zebrafish version are rarely well conserved at protein level (explaining why so few antibodies cross-react) and thus parity of function cannot be assumed, nor will drugs that target human proteins inevitably have the same effect due to different protein sequences. There are few if any pharmacologic studies that express zebrafish proteins and compare drug binding with human, and so it is difficult to be certain what proportion of drugs will reliably target the same proteins in zebrafish. These are important caveats, but generally the more fundamental the mechanism, the better conserved is the genetic machinery, and in the case of evolutionarily ancient processes such as vascular development, there is a high degree of conservation between human and zebrafish [31].

3. Zebrafish studies of induced cancer

A number of studies have successfully induced a range of cancers in zebrafish by genetic modulation or carcinogen exposure. However such approaches take time to induce cancers and often tumours only develop in a proportion of treated fish. For example, melanoma can be induced in genetically modified *mitfa-BRAF*^{V600E}; *p53*$^{-/-}$ zebrafish, but in one study less than 10% of animals developed naevi, and of these only half went on to develop melanoma [32]. Using the same approach a later study showed that around 20% of fish developed melanoma by 15 weeks old and the majority by 20 weeks, although this was accelerated by overexpressing the SETDB1 histone methyltransferase [33]. Mutation of the tumour suppressor *tp53* leads to induction of tumours, but only 28% of fish exhibited cancer by 16.5 months old [34]. This incidence is again increased by further modifying genetic factors; double mutants for *tp53* and the Ewing's Sarcoma gene *ewsa* increased the proportion of fish developing tumours, but these took 20 months to manifest [35]. This model when coupled with the facility to produce transgenic zebrafish allows tracking of melanoma induction from extremely early stages *in vivo*, due to recapitulation of neural crest identity that can be visualised using a *Crestin:GFP* transgenic [Kaufman, 2016 #329]. Other approaches can induce syngeneic tumours reliably and rapidly; genetic activation of hepatic beta-catenin induced hepatocellular carcinoma in the majority of fish by 6 months old [36]. Oncogenic overexpression has been successful in inducing brain tumour formation at even earlier stages [37]. However, although induced tumour formation is an important and potentially more clinically relevant approach for studying cancer in zebrafish, application of *in vivo* imaging is challenging in the ages of fish that develop cancer in such studies, hampering real-time observation of cellular behaviour. For these reasons, and for it's

practical advantages, the majority of studies have applied xenotransplantation of human cancer cell lines, and we will focus on this approach in the remainder of this review.

4. Xenotransplantation of human cancer cell lines

Xenotransplantation plays to the strengths of the zebrafish model system. It is possible to inject hundreds of embryos in a single day, they are remarkably robust even when injected with large tumour burdens, and the use of younger transgenic animals allows full exploitation of the imaging capabilities of the zebrafish, as it provides observation deep within the animal, without obscuring pigmentation or other structures. The use of human lines is of course advantageous for studying behaviour of a characterised and relevant cancer. However, it is important to recognise that questions remain about the degree to which zebrafish models can reproduce the human setting. Poikilothermic zebrafish are usually incubated at 27-28°C, which may affect the behaviour of human cancer lines. It is possible to incubate zebrafish at higher temperatures, though this accelerates embryonic development such that embryos are protected by animal experimentation legislation (in the UK at least) earlier than the 5 dpf cutoff for embryos incubated at normal temperatures.

The tradeoff between the stage of xenograft injection and the ability to perform serial imaging studies means most studies inject cancer cells into early embryos, usually around 2 dpf. Although by this age the circulation is established and there is a reasonably complex cerebral circulation, the trunk vasculature is in the earliest stages of remodelling and growth, and undergoes extensive alterations in the next few days, with lymphatics developing from about 3 dpf [38]. Various sites of injection have been used. Between 2-3 dpf it is straightforward to inject cells either directly into the circulation via the large ducts of Cuvier that provide venous return over the yolk sac or via pericardic and intracardiac

injections. Injection into the duct of Cuvier becomes more challenging in older embryos as this vessel remodels into a smaller deeper vein. The perivitelline space (the gap between the skin and the outer membrane of the yolk sac) is also easily accessed even in older embryos and can accommodate a large volume of tumour cells. This site does not directly communicate with the vasculature but is reasonably close to major vessels and over which vessels grow during development might approximate the peritoneal or pleural cavities, with metastasis possible by either invasion or blood borne spread. Direct injection into the yolk sac itself is technically easy and is another approach of introduction of tumour cells into zebrafish. Due to the small size of the embryo and the relative large size of the needle, injection often results in a leak of cells after injection. Administration of reproducible volumes of cells is therefore challenging, and it is common to screen for and exclude embryos with insufficient tumour loads in order to reduce variability (the large number of embryos that can be generated and injected makes this straightforward).

Although transplantation of cancer cells has been successfully applied in zebrafish at later stages of development (such as juvenile or adult fish), this is technically challenging especially to image. Published studies have applied this to approach to studying the behaviour of human leukemia, prostate, liver [39, 40], breast cancer, fibrosarcoma and melanoma cells [41, 42]. An interesting hybrid approach has been applied successfully by inducing melanoma in zebrafish, which are then cultured and re-injected into both adults and embryos. This elegant model was able to define quantitative metrics describing cancer metastasis[38]. Xenografting human tumour cells into older zebrafish is limited by immune rejection although this can be overcome by irradiation [40, 42], chemical immunosuppression [41] or genetically immunocompromised lines [43, 44]. The use of

immunodeficient or immunosuppressed animals facilitates growth of human cancer cells in both zebrafish and murine models but has limitations such as altered tumour microenvironment. In addition, the growing interest in immune-targeted anti-cancer treatments highlights the importance of using immunocompetent models for cancer research. A novel xenotransplantation model has been developed to circumvent these issues in the adult zebrafish through sequential injection of irradiated then viable tumour cells to induce immune tolerance [39]. This results in an adaptation of the immune system allowing tumour growth and metastasis. This may be applicable to immune-targeted drug discovery studies in zebrafish although the time to achieve immune-tolerance makes high-throughput studies difficult.

Table 2 lists and compares the advantages and disadvantages of zebrafish and mouse xenografting models.

4. Zebrafish xenotransplantation studies investigating metastasis

The following section will summarise the contribution of zebrafish studies to investigate the various stages of metastasis, such as tumour-induced angiogenesis and interactions with endothelial cells, intravasation and extravasation to and from the circulation, dissemination/invasion and tumour formation at a secondary site.

4.1 Tumour-induced angiogenesis

Neoangiogenesis at primary and metastatic tumour sites is a key element of cancer spread and progression. Thus, the process of new vessel formation could present targets for inhibition of tumour recurrence and metastasis. Neoangiogenesis is thought to occur after an “angiogenic switch” is triggered. At this point the cancer colony reaches a size where

perfusion is necessary to provide nutrients and oxygen for growth [45]. A recent study suggests however that endothelial cell-tumour cell interactions at early stages even without blood flow are crucial for tumour progression [46]. Zebrafish xenograft studies showed that microtumours were infiltrated with blood-free 'endothelial cords' for 6-7 days before blood-flow was established. Intriguingly, inhibition of endothelial cord formation and penetration into the tumour resulted in significant reduction in tumour growth. The data suggest that endothelial cells directly stimulated tumour cell proliferation via paracrine signaling (independently of perfusion) which was corroborated through murine experiments. The authors suggest targeting angiocrine signaling between tumour and endothelial cells rather than use of direct anti-angiogenic treatments to reduce unwanted effects on e.g. resistance and invasiveness. A requirement for interactions between cancer and endothelial cells for tumour initiation could also indicate the presence of potential perivascular niches, an environment that promotes tumour cell survival, progression and resistance to therapies [47]. Induction of neoangiogenesis by human cancer cells in zebrafish is dependent on the number of tumour cells and the surrounding microenvironment [48]. Taken together these studies provide evidence that embryonic and adult zebrafish xenograft models may be applied to assess mechanisms of tumour-induced angiogenesis.

Making use of similar models, Nicoli et al. [49] investigated tumour-induced vessel formation and found that cancer cells overexpressing angiogenic factors such as VEGF (vascular endothelial growth factor) or FGF2 (fibroblast growth factor) increased the number of vessels sprouting from the subintestinal plexus into the tumour site. Cells lacking these factors did not induce neoangiogenesis. In addition, newly formed vessels expressed markers of early endothelial development such as VGFR2/KDR, VE-cadherin and Fli-1 suggesting direct interaction between human cancer cells and the zebrafish environment.

Treatment with anti-angiogenic agents inhibited such tumour-induced vascularization. Tumours often encounter hypoxia, which is partly created through the cancer–induced irregular and leaky vascular networks as well as the fast growth of the tumour itself. It has been hypothesised that hypoxia and the associated leaky vessels could be one of the environmental cues for tumour cells to invade areas of healthy vasculature followed by metastasis. To investigate this further, zebrafish exposed to hypoxia have been used to assess whether VEGF and hypoxia promote metastasis of murine fibrosarcoma cells injected into the perivitelline space of 2 dpf zebrafish embryos [50]. Both hypoxia and over-expression of VEGF significantly increased metastasis, while hypoxia induced more tumour vessels compared to normoxia while the primary tumour remained unchanged in size. Inhibition of the VEGF signalling axis in hypoxic and normoxic conditions through sunitinib or VEGFR2-morpholinos blocked invasion and dissemination of tumour cells. The authors concluded that VEGF- and hypoxia-induced neoangiogenesis are crucial events in metastasis. Taken together these studies [49, 50] suggest a crucial role of VEGF signalling in the initial phases of neoangiogenesis and metastasis.

The studies here summarised show that zebrafish models can be successfully exploited for the study of cancer. However, this does not mean that the models currently in use are the most reliable or informative. There are few published comparisons of the behaviour of cancer cells when injected at different densities, sites, ages, or incubation conditions. Rather, most groups understandably rely on a method that works in their hands but varies between different groups and studies. Also, interpretation of results from xenotransplantation is often made more challenging by the lack of control experiments where non-cancer or different cancer cell lines are xenografted. Many studies of tumour

"neovascularisation" do not test whether or not a similar vascular response is elicited by non-cancer cell types for example, meaning their direct relevance to cancer is obscured.

4.2 Intravasation

The study of tumour intravasation is challenging due to the difficulty in identifying the time and site of such relatively rare events. The study of mechanisms of intravasation of tumour cells into blood vessels has thus predominantly relied upon analysis of static images of tumours. However, the imaging capabilities of the zebrafish allow observation and manipulation of tumour intravasation in real-time. A study applying comprehensive high-resolution imaging of tumour-endothelial cell interactions in zebrafish aimed to understand these dynamic communications [41]. Injection of fluorescent human tumour cells into the peritoneal cavity of one month old dexamethasone-treated (to induce immunosuppression) zebrafish induced microtumours at the injection site associated with tumour-induced vascular remodelling and permeabilisation. Tumour cells over-expressing the metastasis gene RhoC preferentially intravasated through the blood vessel wall at active sites of vascular remodelling. Interestingly these sites were governed by the tumour cells themselves through VEGF secretion, with more tumour cells intravasating into blood vessels when both RhoC and VEGF were overexpressed [41]. In addition to tumour and endothelial cell interactions, tumour associated macrophages (TAMs) have frequently been associated with poor prognosis, tumour progression and facilitation of metastasis [51, 52]. The intricate interactions between TAMs and tumour cells, however, remain to be established. Co-injection of TAMs and cancer cells into zebrafish showed that macrophages actively mediated metastases by direct cell-cell interactions [53]. Interestingly most disseminated

tumour cells were closely linked to IL6 and TNFa-activated macrophages in the vasculature. Notably, the pro-tumoural M2-type, in contrast to M1-type macrophages, was found to increase intravasation and metastasis.

4.3 Extravasation

While the early stages of metastasis such as neoangiogenesis and intravasation may be observed in other cancer models, subsequent extravasation from the circulation is more challenging to capture in real time. A study assessing this elusive stage of metastasis detected two separate types of extravasation in zebrafish [54]. RFP-labelled Hela cells were injected into the circulation of 2 dpf *flk1:EGFP* zebrafish embryos and formed emboli-like structures in the caudal artery that extravasated out of the blood stream 17-20h post injection. While some cancer cells actively extravasated via penetration of the endothelial layer others remained as a cluster inside the vessel and were progressively covered by endothelial cells eventually resulting in the exclusion of cancer cells into the surrounding tissue (termed 'covering-type extravasation'). The authors suggested cancer cell-induced activation of endothelial cells could be an additional factor involved in tumour cell extravasation. Silencing of VEGF by siRNA or sunitinib treatment completely inhibited the active/invasive but not the endothelial cell mediated "covering" extravasation type. In a further study aiming to dynamically visualise extravasation, tumour cells injected into the pericardium of zebrafish embryos passively arrested in small capillaries in the head and tail regions after approximately 3-5 hours before actively migrating along the vessel wall [55]. Different cell lines showed variability in their extravasation efficiency, which correlated with their metastatic potential. It was furthermore shown that overexpression of the metastasis related genes twist and VEGFA resulted in increased extravasation while suppression of β_1-

integrin reduced this process. Although direct injection of tumour cells into the circulation does not reflect true metastasis the capability of tumour cells to extravasate and the correlation with their metastatic potential coupled with evaluation of involved factors adds to our understanding of extravasation into new sites.

4.4 Dissemination and homing

Several solid tumours such as breast and prostate show a predilection to metastasise to bone, however, the mechanisms of this directed dissemination and colonisation remains unknown. Existing models allowing analysis of early metastatic events to bone such as murine xenograft models have limitations, especially for large scale studies and *in vivo* tracking of cells over a long period of time. To overcome these limitations Sacco et al. [56] developed a zebrafish xenotransplantation model to investigate multiple myeloma (MM) dissemination and homing to the caudal haematopoietic tissue. The caudal haematopoietic tissue was proposed to comprise a bone marrow-like haematopoietic stem cell niche (HSC niche) and is the area of embryonic haematopoiesis in zebrafish. The HSC niche has previously been suggested to be one of the factors involved in homing of tumour cells to the bone marrow [57]. The authors found intracardiac injection of fluorescently labelled MM cells into 2 dpf *Casper* zebrafish embryos resulted in localisation of the tumour cells to the caudal haematopoietic tissue 30 minutes after injection. Gene expression studies after human exome enrichment of the mixed human-zebrafish sample showed that cells in the CHT area expressed higher levels of IL6-pathway components, adhesion and angiogenesis genes compared to cells outside the caudal haematopoietic tissue. Importantly, silencing of known modulators of MM homing to bone such as CXCR4, VLA4 and FAK or inhibition of the CXCR4 inhibitor by AMD3100 reduced tumour localisation to the caudal haematopoietic

tissue. These results support the relevance of the zebrafish for the study of myeloma homing to the haematopoietic niche, however, more studies are required to validate these findings. A study reporting tumour cell localisation to the caudal haematopoietic tissue area following injection into the duct of Cuvier suggested a direct link between neutrophils (predominantly located at the CHT) and cancer spread [58]. Complete, but not partial, knockdown of neutrophils accompanied by complete knockdown of macrophages blocked tumour cell dissemination to the caudal haematopoietic tissue whereas either induced significant impairment of neovascularisation at the tumour site. However, it was furthermore shown that physiological neutrophil migration occurring between caudal haematopoietic tissue and the tail fin resulted in transient collagen deposits, which in turn appeared to be necessary for tumour cell invasion. Interestingly, VEGFR inhibition reduced neovascularisation and tumour growth at the injection site but induced neutrophil migration, with an associated increase in micrometastases. These studies suggest that dissemination and homing of tumour cells to localised areas in the zebrafish can be detected although direct interactions between fish and human components remain to be established in more detail. A more relevant model to study these aspects of metastases may be introduction of human stromal cells or cytokines to further mimic the human environment.

4.5 Invasion at secondary sites

Zebrafish xenografting has been used to investigate involvement of Smad6-induced inhibition of BMP signalling in breast cancer cell invasion [59]. MCF10A M2 cells showed a clustered invasion phenotype (invasive cells cluster together at similar sites) predominantly at the caudal haematopoietic tissue. The number of clusters was increased by Smad6

overexpression. In contrast, MDA-MB-231 cells injected into the circulation of 2 dpf embryos resulted in isolated single cell invasion in the tail fin. In support of the role of Smad6 in invasion, significantly more cells invaded the tail when Smad6 was overexpressed, but this was reduced when using Smad6-knockdown cells. Smad6 overexpression was associated with inhibition of BMP signalling. The authors went on to investigate the effect of BMP6 pre-treatment on tumour cell invasion in the BMP6-low expressing aggressive MDA-MB-231 cells [59]. Although such pre-treatment did not alter overall invasiveness, these cells adopted a more clustered-phenotype in the tail, indicative of the phenotype of less aggressive MCF10A M2 cells. Interestingly, elevated Smad6 expression in oestrogen receptor negative breast cancer is correlated with poor distant metastasis free survival, leading the authors to conclude that Smad6 and BMP signalling contribute to development of metastatic breast cancer. The same group had shown in an earlier study that the metastatic potential of breast cancer cells in zebrafish was consistent with data from mouse models, indicating the ability of the zebrafish to reproduce data from mammalian studies. In addition, TGF-b signalling has been shown to influence breast cancer metastasis, since inhibiting this signalling cascade reduced cancer cell invasion in the zebrafish [60].

4.6 Assessing metastatic potential in xenografting models

A recent study by El-Nagger et al. [61] demonstrated that Y-box binding protein 1 (YB-1), which regulates HIF1a expression in human sarcoma cells, contributes to increased cell migration in a zebrafish xenograft model. TC32 (Ewing sarcoma) cells were implanted into the yolk sac of 2 dpf casper zebrafish embryos and migration to the tail was detected after 24 hours and quantified up to 120 hours post injection. The active movement of cells into the tail was confirmed by injection of fixed cells or microspheres, which did not migrate.

Silencing of YB-1 using sh-RNA resulted in inhibition of migration to the tail at 120 hours post injection compared to control, suggesting a role of YB-1 on cell motility in cancer cells of mesenchymal origin. This was subsequently confirmed in a murine model. The YB-1-HIF1a signaling cascade may therefore be a treatment strategy effectively targeting early stages of sarcoma metastasis.

Evaluation of the metastatic potential of different human uveal melanoma cell lines showed a similar pattern to that described above [60], with metastatic cell lines being more migratory and proliferative compared with cells from primary tumours [62]. Furthermore, Teng et al. [63] characterised the xenograft zebrafish model in several cancer types including cell lines as well as patient-derived material. For all cell lines the same procedure was used: injection of tumour cells into the perivitelline space of 2 dpf zebrafish followed by quantification of metastatic cells. It was shown that the degree of metastatic potential in zebrafish correlated to invasiveness *in vitro*. Cell lines classed as non-metastatic (for example T47D and HT29) did not metastasise in the zebrafish and chemical or genetic inhibition of invasiveness resulted in reduced spread. Importantly the group also tested the metastatic ability of primary patient derived lung cancer cells suggesting that the model could be used for the evaluation of the degree of aggressiveness in patient samples. The ability to assess patient derived material and potentially to test drug response in a high throughput *in vivo* system could be a promising step towards more personalised treatment strategies, although bioavailability and drug characteristics need to be taken into account.

A further study confirming the feasibility of xenotransplantation of patient material into zebrafish showed that tissue pieces as well as cell suspensions derived from human gastrointestinal tumours (pancreas, colon and stomach carcinoma) were capable of forming metastasis when transplanted into the yolk sac of zebrafish embryos while non-

tumourigenic human tissue did not [64]. Transplantation of primary pancreatic tumour cells into the liver of 5 dpf zebrafish also showed metastatic potential. Interestingly a *cloche* mutant zebrafish line which lacks functional vasculature and circulation showed that transplanted tumour cells into the yolk remained at the site of injection suggesting that a functioning vascular system is required for cell dissemination. A study applying an orthotopic transplantation method suggesting that more relevant tumour cell-microenvironment interactions between donor and host cells could be examined used human glioblastoma cells injected into the zebrafish brain [65]. Although tumour cells invaded the surrounding brain tissue in a calpain-2 dependent manner, tumour cells did not metastasise when injected into the brain or into the yolk sac. Importantly, glioblastoma is known not to spread outside the brain in humans suggesting xenotransplantation into zebrafish preserves the clinical behaviour of this tumour type.

4.7 Cancer stem cells (CSCs) and metastases

Cancer stem cells are considered responsible for drug resistance, tumour propagation and in some cases metastases [66, 67] and are currently extensively investigated in cancer research seeking novel therapeutic targets. Identification of CSCs is performed using a range of molecular and functional assays with the main experimental proof of a CSC phenotype being increased tumourigenicity compared to their non-CSC counterparts when injected into immunocompromised mice [67, 68]. Recent studies have applied the zebrafish model to characterise CSC candidates from breast [69]; leukemia and liver [40] and prostate cancer [40, 70]. Isolated CSC candidates from a breast cancer cell line injected into 2 dpf zebrafish exhibited increased migratory potential and tumour foci formation compared to parental cells [69]. Zoni et al. [70] evaluated the role of miR-25 (a miRNA absent from the CSC

population in prostate cancer cell lines and patient material) on invasiveness of human prostate cancer stem like cells. Prostate cancer cells overexpressing miR-25 or control were injected into the circulation of 2 dpf zebrafish embryos. While control cells readily extravasated from the vasculature into the caudal haematopoietic tissue area this was significantly reduced in miR-25 overexpressing cells. This reproduced *in vitro* data showing a miR-25-dependent negative regulation of a metastatic phenotype. These studies show that zebrafish may be used to study tumourigenicity and metastatic potential of isolated CSCs in a convenient and high throughput model, however, further studies including the use of CSCs isolated from patient material are necessary to further help characterise the putative cancer stem cell.

5. Evaluation of anti-metastatic treatments in zebrafish

The zebrafish is an excellent model to observe intricate cell-cell interactions during metastasis but could also be applied for drug discovery which has been reviewed in detail elsewhere [71, 72]. Although we earlier highlighted the fact that drug effects should not be assumed to be conserved between human and zebrafish due to protein differences, there is more than enough evidence to show that existing drugs do often work in zebrafish, and that zebrafish can be used to identify novel bioactive molecules. Several studies have investigated the inhibition of metastasis through targeted disruption of molecules and proteins involved in tumour cell spread. Inhibition of the PDK1/PLCγ1 complex implicated in invasion and metastasis [73] using the small molecule inhibitor 2-O-Bn-InsP_5 significantly reduced dissemination of MDA-MB-231 breast cancer cells in 2 dpf *Tg(kdrl:HsHRAS-mCherry)*^{s896} zebrafish embryos [74]. In this study two different tumour cell injection

methods were used, directly into the circulation via the duct of Cuvier or the perivitelline cavity. Both methods showed significant reductions of disseminated tumour cells after 2-O-Bn-InsP_5 treatment compared with control. The choice of zebrafish as *in vivo* model for metastasis and subsequent verification of drug efficacy provided the first evidence of novel anti-metastatic treatment targeting the PDK1/PLCγ1 signalling pathway in breast cancer [74] or by using the SIRT1/2 inhibitor Tenovin-6 in Ewing sarcoma [75]. Another study aimed to disrupt the dissemination of tumour cells by blocking av-integrins either by genetic or chemical interference using the small molecule antagonist GLPG0187, a non-peptide RGD antagonist designed to inhibit av-integrin interactions [76]. Knockdown of av-integrin in MDA-MB-231 cells resulted in significantly lower dissemination and metastasis in zebrafish embryos suggesting that the av-integrin is involved in cancer dissemination. Chemical inhibition using GLPG0187 also reduced metastasis in zebrafish treated either prior to or after tumour implantation. Dose-dependent inhibition of tumour cell dissemination in zebrafish was reported after treatment with the resveratrol analogue DHS (4,4-dihydroxy-trans-stilbene) [77]. In contrast to the anti-metastatic effect in zebrafish and in a murine liver metastasis model, lung metastases in mice were not significantly reduced after DHS treatment. The study highlights the importance of understanding the bioavailability of the investigated drug in each model and different tissues since in the mouse insufficient DHS or only inactive forms were able to reach the lungs.

Anti-angiogenesis treatments are often applied to target cancer progression and metastasis. A *de novo* design method to find drugs targeting VEGFR2 identified the compound SKLB1002 [78]. Administration to zebrafish showed inhibition of intersegmental vessel formation and inhibition of tumour-induced neoangiogenesis at the injection site (murine B16-F10

melanoma cells implanted into perivitelline space of 2 dpf zebrafish embryos). Inhibitory results on vasculature were comparable to the anti-angiogenic compounds sunitinib and vandetanib. A recent study investigated whether zebrafish could be used to validate anti-angiogenic potential of miRNAs [79]. Prostate cancer cells (DU-145) were transfected with miRNAs before implantation into the perivitelline space of 48hpf zebrafish embryos resulting in inhibition of neovascularisation compared to control for selected miRNAs. Further studies examined a number of anti-angiogenic compounds on glioma [80] and breast cancer [81] cell induced neoangiogenesis in zebrafish embryos further adding evidence that the model can be used for drug evaluation studies.

6. Conclusion

The studies summarised in this review show that the zebrafish is a useful model of cancer and metastasis, whose unique advantages allow direct observation of cellular behaviour and novel mechanistic insights. We have sought to balance these advantages with a clear discussion of the caveats and disadvantages of this model such that a non-zebrafish researcher can assess whether or not the model is suitable to test specific scientific questions.

Although not yet well-established as a model of anti-cancer drug discovery, for candidate drug testing the zebrafish has already shown great promise, and the number of reports using this model seem likely to increase as familiarity with the model increases.

7. Expert opinion

The societal and economic impacts of cancer and metastasis cannot be overstated, and there exists no single ideal model organism with which to study these diverse diseases. Exploitation of a range of model organisms and approaches is thus justified, and zebrafish possess particular advantages, particularly in the areas of *in vivo* imaging, and genetic tractability, coupled with lower costs than higher organisms. The most suitable model for a particular study continues to be determined by the exact scientific question to be answered, but the relevance and generalizability of any findings are greatly increased where observations made in one model are reproduced in other systems. For these reasons, we see the zebrafish as complementary to existing model systems. It is likely that most impact will come from studies that apply a range of models to answer a particular question, and it remains essential that where possible we need to closely reference our findings to what is observed in human cancers if we hope to achieve therapeutic breakthroughs.

Xenotransplanation studies in zebrafish have already proven to be extremely informative but it is important to appreciate the multiple genetic, molecular, and physiological differences inherent in this organism. Xenotransplantation can only study relatively small numbers of cancer cells, which may mean that mimicking the tumour microenvironment in larger human tumours, particularly with regard to such elements as tissue hypoxia, is more challenging. Although this review has focused on xenotransplantation, we do not wish to underplay the importance of induced tumour studies in zebrafish. These are necessarily more complex than xenotransplantation, and have some technical and practical disadvantages, but are potentially more clinically relevant and allow investigation of tumour initiation and other areas not possible with xenografting.

Where zebrafish studies seem to have most to contribute is their ability to provide observations of cancer cell behaviour and metastasis in a manner impossible in other

models, either because current microscopy cannot visualise the tissue sufficiently in other models, or because instrumentation prevents longer-term imaging. For these purposes, zebrafish excel and have the real potential to uncover previously unsuspected interactions between cancer cells and the host. The current rate of technological advances in microscopy, coupled with the fact that techniques for genetic manipulation in zebrafish is proceeding at a pace that will probably soon allow tissue-specific, conditional approaches make us confident that we will be able to use the zebrafish to study *in vivo* cancer cell behaviour at a resolution that would not have been dreamt of a decade ago.

Although technically an *in vivo* model, some xenotransplantation studies in zebrafish use the fish as little more than a sophisticated culture dish, and the additive benefit of this approach is possibly limited. Although such studies are still of course valuable, to justify the additional technical challenges and animal welfare considerations zebrafish studies should observe the behaviour of the host cells and tissues as closely as the cancer cells themselves. Many studies do not present data on metrics such as heart rate, animal growth, or blood flow that are necessary to understand whether cancer cell behaviour is being directly affected by for example a drug treatment, or whether the drug simply induces general toxicity that indirectly affects the tumour.

Although an increasing number of zebrafish-based small molecule drug screens have been published, the likelihood of such screens being useful for cancer studies for unbiased screens seems low. To screen thousands of molecules requires a simple and highly reproducible assay, and the technical challenges of cancer xenotransplantation mean this is unlikely to be possible, particularly since cell-based drug screens are readily possible for many cancer-related mechanisms. Where drug screening in zebrafish is more likely to be useful is as an intermediate step in which to test hits from *in vitro* screens prior to more

expensive and time-consuming rodent studies. Although the lack of protein conservation between human and zebrafish makes certainty about drug effects on the host tissue difficult, most fundamental mechanisms that govern cell behaviour or vascular development appear well enough conserved to allow a reasonable expectation that hits from human cell based screens will exert effects in zebrafish based assays. This is of course helped when the injected cancer cells are of human origin themselves and the drug target is expressed on the cancer rather than the host.

The literature summarised in this review shows that human cancer cells when introduced into zebrafish embryos behave in a way that reproduces clinical features of human cancers, is modulated by interventions known to influence cancer clinically, and leads to novel observations that are biologically plausible and have in some cases been validated in mammalian models. For these reasons we are reasonably confident that within our careers we will see clinical introduction of drugs that target molecules or mechanisms that have been discovered using zebrafish models of cancer.

Article highlights box

- Zebrafish allow unrivalled in vivo imaging of cellular behaviour thanks to optical clarity and a range of tissue specific transgenic lines.
- An increasing number of zebrafish cancer studies have now been reported, most using xenotransplantation of human cancer cell lines.

  Mechanisms known to drive metastasis in human and rodent models appear conserved in the zebrafish, and novel mechanisms of cancer metastasis discovered in zebrafish appear to be reproduced in rodents.

  The ability to treat zebrafish by immersion in the drug greatly facilitates testing of drug effects, and such studies suggest drugs discovered in zebrafish are likely to be effective in mammalian models.

  Although it is too early to be certain, it seems likely that the zebrafish will contribute novel insights that ultimately lead to patient benefit.

Table 1 An overview of zebrafish xenotransplantation studies assessing metastatic processes and summarises the injection methods, cancer cell type and zebrafish lines used.

Cancer cell line/origin	Zebrafish model (age at injection, strain)	Injection site	Ref
Mouse melanoma (B16, B16F10), colon carcinoma (CT26), mammary gland tumor (4T1); human breast cancer (MCF7, MDA-MB-231)	2dpf, *Tg(flk1:EGFP)*	Pericardial cavity, into circulation from the perivitelline space	[48]
Tumour cell lines (MM.1S, MM.1S/GFP+, BCWM.1, HeLa, MDA-MB-231); primary human $CD138^{+}$ multiple myeloma cells	2dpf, Casper (albino)	Direct intra-cardiac injection	[56]
Human fibrosarcoma (HT1080), breast cancer (MDA-MB-231, MDA-MB-435), colon adeno-carcinoma (SW480, SW620)	2dpf, *Tg(fli1:EGFP)*	Common cardinal vein	[55]
HeLa cells	2dpf, *flk1: EGFP*, dexamethasone for 5-7h	Common cardinal vein	[54]
Mouse aortic endothelial (MAE), tumorigenic FGF2-overexpressing FGF2-T-MAE, breast cancer (4T1); human breast cancer (MDA-MB-231), prostate cancer (PC3); zebrafish fibroblast cell line (ZF4/PAC2)	2dpf, *Tg(fli1:GFP), Tg(mpx:GFP)*	Approximately 60 µm above the ventral end of the duct of Cuvier	[58]
Human prostate cancer (PC-3M-Pro4)	2dpf, *Tg(mpo:GFP)^{i114}*	Approximately 60 µm above the ventral end of the duct of Cuvier	[70]

Human cancer cell lines (293T, 3T3, and MDA-MB-231), MCF10A-derived breast epithelial cell lines M1, M2, M4	2dpf, *Tg(fli1:GFP)*	Duct of Cuvier	[60]
MCF10A M2, MDA-MB-231	2dpf, *Tg(fli1:GFP)*	Duct of Cuvier	[59]
Primary gastrointestinal tumours and control cells; human pancreatic tumour cell lines (PaTu8988-S, PaTu8988-T); mouse mammary epithelial cells (EpH4) transformed with oncogenic Ras (EpRas)	2dpf, albino, *Tg(fli1:eGFP)*, cloche	Tissue pieces or cell suspensions into the yolk sac, liver	[64]
Human glioblastoma (U87MG)	4dpf for tropical 5D, 10dpf for *Tg(fli1:egfp)*	Yolk sac, brain	[65]
Human breast carcinoma (BT-474)	2dpf, wild type AB	Yolk sac	[69]
Human leukemia (K562), prostate (DU145), liver (HepG2)	Embryo: 2dpf, Adult: 20-Gy ionizing radiation, *nacre/rose/fli1:egfp*	Embryo: avascular region of the yolk sac Adult: near dorsum aorta	[40]
Human uveal melanoma (Mel270, OMM2.3 and OMM2.5, 92.1, OMM1)	2dpf, *TG(fli1:EGFP)*	Yolk sac	[62]
Human prostate cancer (PC-3), chronic myelogenous leukemia (K562), hepatocarcinoma (HepG2)	2dpf, adult, *nacre/rose/fli1:egfp*	Embryo: avascular region of the yolk sac Adult: near dorsal aorta	[39]
Mouse aortic endothelial (MAE), tumorigenic FGF2-overexpressing FGF2-T-MAE, melanoma (B16-BL16); human adenocarcinoma (Tet-FGF2), ovarian carcinoma (A2780), breast carcinoma (MDA-MB-435)	2dpf, wild-type AB and *VEGFR2:G-RCFP*	Perivitelline space between yolk and periderm (duct of Cuvier area), close to subintestinal vessels	[49]
Mouse fibrosarcoma (T241), Lewis Lung Carcinoma (LLC); human breast cancer (MDA-MB-231), ovarian cancer cells	2dpf, *Tg(fli1:EGFP)*	Perivitelline cavity	[50]

(OVCAR 8)			
Mouse melanoma cells (B16), colon cancer (CT26); human embryonic kidney cells (HEK293)	2dpf, *Tg(flk1: EGFP)*	Perivitelline space	[82]
Human breast cancer (MDA231, T47D) MCF10A, prostate cancer (DU145, LNCaP), colon cancer (SW620, HT29), pancreatic ductal adenocarcinoma (ASPC-1, BxPC3)	2dpf, wild-type AB or *Tg(kdrl:EGFP)*	Perivitelline space	[63]
Mouse Lewis lung carcinoma (LLC), fibrosarcoma (T241); human ovarian cancer (OVCAR8)	2dpf, *Fli1:EGFP*	Perivitelline space	[53]
Melanoma: mouse (B16), human (A375)	2dpf, *Tg(flk1:eGFP), Tg(flk1:mCherry), Tg(Gata1:DsRed), Tg(fli1a:GFP), Tg(lysozymeC:GFP) Tg(MPO:GFP), p53* & *cloche* mutant	Perivitelline space	[46]
Human breast carcinoma (MDA-435), fibrosarcoma (HT1080), melanoma (B16)	25–35 days old, AB or T*g(fli1:egfp),* 10g/ml dexamethasone for 2 days	Peritoneal cavity	[41]

Table 2: Advantages and disadvantages of mouse and zebrafish experimental models

	Advantages	Disadvantages
ZEBRAFISH	- Small size and easy to maintain large numbers at a low cost - Proliferate easily: over 200 embryos per pairing - Embryonic development is ex-utero facilitating the transplantation of tumour cells at distinct stages - Optically transparent embryo and adult transgenic lines, allowing for non-invasive visualisation. Repeated imaging of tumour development *in-vivo*. Imaging at single cell level possible. - Use of transgenic lines facilitates studies on interactions with human cells and specific host factors - No immune rejection in early transplantation settings - Zebrafish are permeable to small molecules through immersion enabling high throughput screening of drug efficacy and toxicity	- Rapid and continuous development of early embryonic stages might restrict cancer studies - Anatomical differences with mammals make certain tumour models impossible to develop such as lack of mammary and prostate glands, joints, limbs, and lungs - Since zebrafish are poikilothermic, studies where homeostatic temperatures are required might be affected - Antibodies against zebrafish markers are limited - Only specific stages of the metastatic cascade can be visualised - Immune suppression required to grow xenografts in adult stages - Degree of interaction between zebrafish and human cells not well established - Transplanted cells are exposed to a different host niche and different environmental factors than humans
MOUSE	- Xenograft models are more advantageous in determining efficacy of novel drugs and chemotherapeutic responses - Tracing and imaging of tumours can be done for longer time periods than in zebrafish - Tumour microenvironment can be manipulated into a close representation of a human (humanized mice) - Well established tumour models are available for a range of cancer types	- Higher cost of maintaining and carrying out large-scale experiments compared to zebrafish - The complete process of tumour progression and metastasis can take up to several weeks/months to study - *In vivo* imaging of metastatic tumours often requires sacrifice of the animal. High resolution longitudinal imaging challenging - Immunocompromised mice are usually required for tumour xenograft models, which may not recapitulate the crosstalk between the tumour and the immune system - Transplanted cells are exposed to a different host niche and different environmental factors than humans

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