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Review

Zebrafish embryo as a tool to study tumor/endothelial cell cross-talk☆

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ABSTRACT

Tumor/endothelial cell cross-talk plays a pivotal role in the growth, neovascularization and metastatic dissemination of human cancer. Recent observations have shown that the teleost zebrafish (*Danio rerio*) may represent a powerful experimental platform in cancer research. Various tumor models have been established in zebrafish adults, juveniles, and embryos and novel genetic tools and high resolution in vivo imaging techniques have been exploited. In particular, grafting of mammalian tumor cells in zebrafish embryo body may simulate early stages of tumor development, neovascularization, and local invasion whereas the injection of cancer cells in the bloodstream of zebrafish embryo may allow the study of metastatic homing and colonization. This review focuses on the recent advances in tumor xenotransplantation in zebrafish embryo for the in vivo study of the cancer neovascularization, invasion and metastatic processes. This article is part of a Special Issue entitled: Animal Models of Disease.

1. Introduction

The teleost zebrafish (*Danio rerio*) has exceptional utility as a human disease model system and represents a promising alternative platform in cancer research [1].

As other teleost fishes, zebrafish spontaneously develops a wide variety of benign and malignant tumors in virtually all organs, with characteristics that resemble those of human tumors [2]. Also, several approaches have been attempted to induce cancer in zebrafish (reviewed in [3]). In a first set of experiments tumor formation was induced by exposure to chemical carcinogens (e.g. diethylnitrosamine, N-methyl-N′ nitro-N-nitrosoguanidine, and 7,12-dimethylbenz[a]anthracene). Adult fishes developed a wide range of tumors in different organs including liver, gill, gastrointestinal tract, pancreas/kidney, testis, muscle, skin and vasculature [2,4,5]. Next, forward genetic screening, target-selected inactivation of tumor suppressor genes, and tissue-specific expression of mammalian oncogenes were used to investigate the molecular bases and biological features of different tumor types in zebrafish adults [6–10]. For instance, injection of the rag2-KRASG12D construct into one-cell-stage embryos represents a model of pediatric rhabdomyosarcoma in zebrafish [8]. This model was recently used to identify tumor propagating cells and to define the functional consequences of tumor cell heterogeneity of this type cancer in zebrafish adults [11]. Finally, permanent gene inactivation by "targeting-induced local lesions in genes" (TILLING) [12] and zinc-finger nuclease [13] approaches have been utilized to assess the impact of target genes on different aspects of tumor biology in adult zebrafish.

When compared to these tumor models, direct transplantation of tumor cells in zebrafish may represent a useful approach to investigate the role of host microenvironment in the early phases of tumor growth, including angiogenesis, local invasion and metastatic spreading. To this aim, several studies have shown the feasibility of injecting mammalian tumor cells in zebrafish adults, juveniles and embryos. Differences in body size and transparency, immune system functionality, genetic tools, drug delivery and bioavailability represent only some of the major differences that should be considered when investigating tumor grafting in zebrafish at different stages of development [14,15].

This review describes the results obtained with recently developed tumor graft models in zebrafish, with particular emphasis to tumor xenotransplantation models in zebrafish embryos for the in vivo study of cancer neovascularization, invasion and metastatic processes.

2. Tumor transplantation in zebrafish adults and juveniles

Transplantable tumor cell lines have been generated in clonal zebrafish and maintained for several passages in syngeneic and isogeneic adults [16]. Microarray analysis has shown that gene expression signatures are conserved in fish tumors when compared to their human counterpart [1]. Also, limiting dilution cell transplantation experiments in irradiated zebrafish adults have provided valuable information about cancer stem cell self-renewal in both leukemias and solid tumors (reviewed in [17]). Relevant to tumor studies in zebrafish adults, a transparent *casper* zebrafish line that lacks all types of pigments has been generated, allowing the rapid identification of transplanted tumor cells [18]. Crossing of the *casper* mutant with

Abbreviations: GFP, green fluorescent protein; FGF2, fibroblast growth factor 2; hpf, hour post fertilization; ISV, intersegmental vessel; MO, morpholino oligonucleotide; SIV, subintestinal vein; VEGF, vascular endothelial growth factor; ZFYM, zebrafish yolk membrane

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transgenic lines that label vasculature or internal organs with fluorescent tags or harbor transgenic-induced tumors represents useful approaches to study tumor–host interactions in zebrafish adults with different optical platforms [19–22].

Noninvasive imaging in non-transparent zebrafish adults has been also attempted. Ultrasound biomicroscopy has been used to follow the growth of liver tumors, their vascularity, and response to treatment [23]. Other imaging techniques, including microcomputerized axial tomography, micromagnetic resonance imaging, and optical projection tomography can be applied in zebrafish and will help to study tumor grafts in adult zebrafish [24].

Due to the smaller size, zebrafish juveniles may represent an interesting alternative to adult animals to investigate tumor cell behavior by high resolution in vivo imaging techniques. To this aim, human cancer cells have been successfully transplanted in the peritoneal cavity of 30 day-old *casper* zebrafish mutants [25]. Since juvenile zebrafish has a functional immune system, dexamethasone administration was required to prevent the rejection of the tumor cell engraftment. This has allowed the study of the dynamics of microtumor formation and neovascularization, leading to a detailed description of the interaction among fluorescent tumor cells and the green fluorescent protein (GFP)-labeled vasculature of the host by three-dimensional reconstruction of confocal microscopy images. The results of these studies have shown that tumor cells secreting human vascular endothelial growth factor (VEGF) promote fish vessel remodeling and angiogenesis and that the human metastatic gene *RhoC* drives the initial steps of the metastatic process.

When compared to zebrafish embryos (see below), the impact of the tumor graft on the mature vasculature of juvenile fishes may recapitulate more closely the events that occur during tumor angiogenesis in adult animals and cancer patients. Indeed, developing vessels of zebrafish embryos may respond differently to tumor grafts compared to the fully developed vasculature of juvenile animals [26]. However, at variance with zebrafish embryos, the potent antisense morpholino oligonucleotide (MO) gene targeting approach [27] is unfeasible in zebrafish juveniles.

3. Tumor transplantation in zebrafish embryos

When compared to other vertebrate model systems, zebrafish embryos offer many advantages, including ease of experimentation, drug administration due to their permeability to small molecules, and amenability to in vivo manipulation. Also, as stated above, zebrafish embryo is suitable for transient gene inactivation via MO injection [27]. Experimental evidences indicate that zebrafish embryo allows disease-driven drug target identification and in vivo validation, thus representing a powerful bioassay tool for small molecule testing and dissection of biological pathways alternative to other vertebrate models [28]. Relevant to this point, zebrafish embryo is suitable for high-throughput screening of chemical compounds using robotic platforms [29]. Also, the use of gold nanobubbles for mechanical tumor ablation [30] and of single-walled carbon nanotubes for the local delivery of anti-angiogenic agents like thalidomide [31] indicates the possibility to utilize zebrafish for investigating the efficacy of novel nanotechnological approaches for cancer therapy.

On these bases, tumor transplantation in zebrafish embryos may represent a simple and rapid approach to study tumor/endothelial cell cross-talk during neovascularization, tumor cell invasion and metastasis. Indeed, the optical transparency and ability to survive for 3–4 days without functioning circulation make the zebrafish embryo highly amenable for tumor/vascular biology studies. External fertilization allows the possibility to transplant tumor cells at specific developmental stages starting from the blastula stage to 48 h post fertilization (hpf) [32]. Also, because of the immaturity of the immune system in zebrafish embryos, no xenograft rejection occurs at this stage with no need for immune suppressing agents [14,33]. Many embryos can be injected by a single operator in a few hours improving the validity of statistical analysis. In a few days tumor cells transplanted into different anatomical sites (e.g. blastodisk, yolk sac, hindbrain ventricle, and bloodstream) can develop tumor masses, providing useful information about the aggressiveness of the disease and the role of specific genes in tumor dissemination and metastasis formation [34].

The injection of human melanoma cells in zebrafish embryos at the blastula stage represented the first attempt to explore the potential bidirectional interactions between cancer cells and embryonic stem cell microenvironment [35]. The results indicate that developing zebrafish can be used as a biosensor for tumor-derived signals. However, grafting of tumor cells at this stage, well before vascular development, results in their reprogramming toward a non-tumorigenic phenotype, thus hampering any attempt to investigate tumor-driven vascularization. At variance, injection of melanoma cells into the hindbrain ventricle or yolk sac of 48 hpf embryos results in the formation of tumor masses within 4 days [36]. Immunostaining analysis of the grafts revealed the presence of blood vessels within the brain and abdominal lesions, even though the high vascularity of the invaded regions did not allow easy discrimination between developmental and tumor-induced angiogenesis [36]. Together, these results underlie the possibility to use zebrafish embryos to investigate the mechanisms and biological implications of tumor/host cell cross-talk in cancer biology.

3.1. Tumor angiogenesis in zebrafish embryos

Angiogenesis, the process of new vessels formation from pre-existing ones, plays a key role in tumor growth and metastasis [37]. Thus, the identification of anti-angiogenic drugs and of angiogenesis-related targets has significant implications for the development of anti-neoplastic therapies [38,39]. Various animal models have been developed in rodents and in the chick embryo to investigate the angiogenesis process and for the screening of pro- and anti-angiogenic compounds, each with its own unique characteristics and disadvantages [40]. To this respect, tumor cell engrafting in zebrafish embryos may provide a valid alternative to other in vivo vertebrate animal models [41,42].

The basic vascular plan of the developing zebrafish embryo shows strong similarity to that of other vertebrates [43]. At the 13 somite-stage, endothelial cell precursors migrating from the lateral mesoderm originate the zebrafish vasculature and a single blood circulatory loop is present at 24 hpf. Blood vessel development continues during the subsequent days by angiogenic processes. In particular, angiogenesis occurs in the formation of the intersegmental vessels (ISVs) of the trunk that will sprout from the dorsal aorta at 20 h. Also, the subintestinal vein vessels (SIVs) originate close to the duct of Cuvier area at 48 hpf and will form a vascular plexus across most of the dorsal–lateral aspect of the yolk ball during the next 24 h [43].

Previous studies had shown that developmental angiogenesis in the zebrafish embryo, leading to the formation of the ISVs of the trunk [44] and of the SIV plexus [45], represents a target for the screening of anti-angiogenic molecules [46,47]. In these assays, low molecular weight compounds dissolved in fish water are investigated for their impact on the growth of new blood vessels driven by the complex network of endogenous, developmentally regulated signals. More recently, a zebrafish yolk membrane (ZFYM) assay has been proposed based on the injection of an angiogenic growth factor [e.g. recombinant fibroblast growth factor-2 (FGF2)] in the perivitelline space of zebrafish embryos in the proximity of developing SIVs [48]. FGF2 induces a rapid and dose-dependent angiogenic response from the SIV basket, characterized by the growth of newly formed, alkaline phosphatase-positive blood vessels [48]. The ZFYM assay differs from the previous zebrafish-based angiogenesis assays since the angiogenic stimulus is represented by a well-defined, topically delivered exogenous agent that leads to the growth of ectopic blood vessels. This allows the screening of low and

high molecular weight antagonists targeting a specific angiogenic growth factor and/or its receptor(s) [48].

However, the study of vascular development and on the effects of positive or negative modulators of the embryonic angiogenic process may have important limitations when translated to cancer research. Indeed, tumor-induced vessels show profound morpho-functional alterations when compared to the normal vasculature [37]. This is reflected by significant differences in gene expression profiling between normal and tumor-derived endothelium [49,50]. Thus, models based on tumor cell transplantation in zebrafish embryos appear to be more suitable for studying the tumor-driven angiogenesis process and its modulators. To this respect, we [41,51] and others [36,52] have characterized the angiogenic response elicited by different mammalian tumor cell lines transplanted into the perivitelline space of 24 hpf-old or 48 hpf-old embryos in the proximity of the developing SIV plexus. Pro-angiogenic factors released locally by the tumor graft affect the normal developmental pattern of the SIVs by stimulating the migration and growth of sprouting vessels towards the implant. One to two days after tumor cell grafting, whole mount phosphatase alkaline staining allows the macroscopic evaluation of the angiogenic response. The use of transgenic zebrafish embryos, in which endothelial cells express GFP under the control of endothelial-specific promoters ([53] and references therein), allows the observation and time-lapse recording of newly formed blood vessels in live embryos by epifluorescence microscopy and by in vivo confocal microscopy [41,51] (Fig. 1A). Also, quantum dots may be used as labeling agents of the zebrafish embryo vasculature for long-lasting intravital time-lapse studies [54]. This model allows the possibility to investigate directly and with high resolution the dynamics of tumor neovascularization as the result of new sprouts from the host vasculature and its origination from $VEGFR2^{+}$ individual endothelial cells. Vessel sprouts connect to each other to form endothelial loops that are then accumulated into the new irregular tumor vasculature [55].

The identification of genes essential for blood vessel formation is of pivotal importance for the understanding of the angiogenesis process and for the discovery of novel therapeutic targets. In zebrafish embryos, MO injection induces a transient translational block in gene function [27]. Gene inactivation by this approach is easy and fast (3–4 days) when compared to the generation of knock-out mice (several months). Also, the simultaneous injection of different MOs may allow the inactivation of more than one gene at the same time. This represents a paramount advantage compared to any mammalian assay available and it can be exploited for the identification of novel gene(s) involved in tumor neovascularization. Accordingly, we have shown that MO-induced inactivation of *VE-cadherin* [41] or *calcitonin receptor-like receptor* [56] zebrafish gene orthologs results in a significant inhibition of the angiogenic process triggered by the tumor graft in zebrafish embryos. Similarly, silencing of the LIM domain kinases *LIMK1* and *LIMK2* inhibits neovascularization induced by human pancreatic tumor cells grafted in zebrafish embryos [57].

As stated above, because of its permeability to small molecules, zebrafish embryos can be used for the screening of novel angiostatic drugs [28]. Accordingly, systemic exposure of live zebrafish embryos to anti-angiogenic compounds dissolved in fish water results in a significant inhibition of neovascularization triggered by various murine and human tumor grafts (Table 1). Thus, tumor cell engrafting in zebrafish embryo represents a short-term in vivo assay suitable for the identification of tumor angiogenesis inhibitors.

3.2. Tumor invasiveness and metastasis in zebrafish embryos

The vast majority of all cancer deaths are caused by metastatic expansion of primary tumors as a consequence of cancer cell dissemination into the body mainly via the lymphatic and blood vessels [58]. The process that leads to the formation of cancer metastases consists of a long series of sequential, interlinked and selective steps that include local invasion, intravasation, arrest in distant capillaries, extravasation, and colonization [59]. During this process, a permissive microenvironment is crucial for metastasis development, underlying the importance of tumor/host cell cross-talk in cancer progression [60]. Also, because of the heterogeneity of cancer cells in primary tumors and metastases, and because of the organ-specific microenvironment, metastatic cells may become in many cases resistant to conventional cancer therapies [59]. Moreover, preclinical studies have shown that the benefits due to anti-angiogenic therapy with VEGF-pathway-inhibitors might be offset by increased tumor invasiveness and augmented metastatic potential [39,61]. Thus, a better understanding of the pathogenesis of the metastatic process at systemic, cellular and molecular levels is essential for the design of new, more efficacious therapeutic strategies.

Due to its transparency, zebrafish embryo allows the study of the behavior of tumor cells grafted in the embryo body or blood stream by high resolution in vivo imaging techniques. In a first set of experiments, Marques et al. [62] injected fluorescently labeled mouse mammary epithelial cells transformed with oncogenic Ras into the yolk sac of 2 day-old zebrafish embryos. Using the transgenic zebrafish line Tg(fli1:eGFP) that exhibits a green fluorescent vasculature, the local invasion of tumor cells, their circulation into the blood stream, extravasation and colonization at distant sites could be followed by time-lapse microscopy. Also, explants from gastrointestinal primary human tumors grafted into zebrafish embryos were able to induce micrometastasis formation within 24 h after transplantation whereas non-tumor tissues were ineffective. Furthermore, human tumor primary cells organotopically implanted in zebrafish liver showed invasiveness and metastatic behavior [62]. Together, these results suggest that zebrafish embryo may represent a model for the rapid analysis of the metastatic behavior of primary human tumor specimens.

Zebrafish xenografts have been used to assess the role of pro-metastatic miR-10a [63] as well as of *Y-box binding protein 1* [32] and *ribosomal S6 kinase* [64] genes and of the ATP-gated $P2X_7$ receptor [65] in tumor invasion. The mechanism of local invasion of tumor cells was investigated further by Lal et al. [34] following the orthotopic injection of human glioblastoma cells into the brain of zebrafish embryos. By 6 days after injection, glioblastoma cells infiltrate the brain, elicit an angiogenic response, and closely align along the abluminal surface of brain blood vessels that may serve as migratory tracks for tumor cells as it occurs in human primary tumors. Interestingly, glioblastoma cell infiltration and vessel interaction were both attenuated by knockdown of the calcium-activated protease calpain 2. In keeping with these observations, grafting of a limited number of highly metastatic murine melanoma B16F10 cells or human breast carcinoma MDA-231 cells onto the inner surface of the pericardial membrane of zebrafish embryos at 48 hpf causes the co-option of host ventral aorta [66]. Then, tumor cells move longitudinally along the aorta that is used as a migratory track. Similarly, time-lapse imaging revealed that single tumor cells injected into the trunk tissue migrate rapidly towards the nearest ISV, thus underlining the tight cross-talk between tumor and endothelial cells during the invasive phase of the metastatic process [66].

Vessel co-option is followed by an angiogenic response that leads to neovessel formation. Hypoxia represents an important driving force for tumor angiogenesis, mainly mediated by VEGF upregulation via activation of the hypoxia inducible factor (HIF) signaling pathway [67]. When metastatic murine and human cancer cells are injected into the perivitelline cavity of 48 hpf zebrafish embryos maintained in hypoxic water (7.5% air saturation), invasion into neighboring tissues, dissemination, and metastasis of labeled tumor cells was greatly enhanced when compared to cells injected under normoxic conditions [68]. Consistent with increased tumor cell dissemination, hypoxia significantly stimulated neovascularization and tortuosity of the tumor vasculature via tumor cell-derived VEGF upregulation. Of note, VEGF receptor blockade by sunitinib administration in the fish water or by MO injection inhibited hypoxia-mediated pathological angiogenesis as

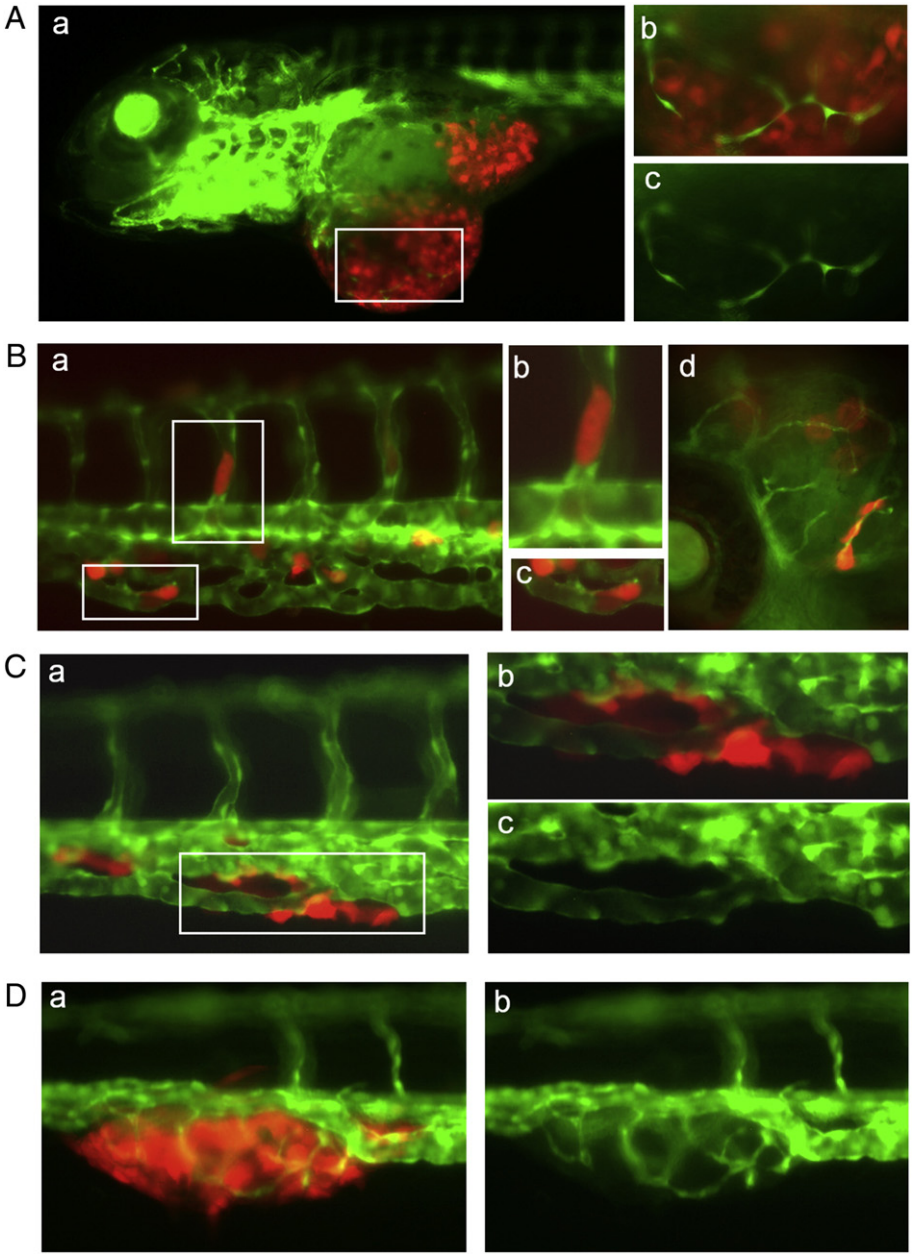

Fig. 1. Tumor xenografts in zebrafish embryo. Labeled murine melanoma DsRed-B16-BL6 cells were injected in circulation in the duct of Cuvier of transgenic tg(fli1:EGFP)^{y1} zebrafish embryos (80–100 cells/embryo) at 48 hpf. Then, embryos were analyzed by fluorescence microscopy. A) Neovascularization of tumor graft. Four days post injection (dpi), a DsRed-B16-BL6 graft (in red) has induced a neovascular response from the SIV plexus (zebrafish endothelium in green) (a). Boxed area is shown at higher magnification in panels b and c. The red channel image was omitted in panel c to highlight the newly formed microvascular network. B) Tumor cell arrest in embryo vasculature. Three hours post injection (hpi) in the blood stream, DsRed-B16-BL6 cells arrest in ISVs and tail vascular plexus (a, the same cells are shown at higher magnification in panels b and c, respectively) and in the brain vasculature (d). C) Extravascular micrometastases in zebrafish embryo. At 4 dpi, tumor cells have formed extravascular micrometastases in the tail vascular plexus (a). Boxed area is shown at higher magnification in panels b and c. The red channel image was omitted in panel c to highlight the extracellular localization of tumor cells. D) Neovascularization of tumor micrometastases. At 5 dpi, a DsRed-B16-BL6 micrometastasis has induced a neovascular response in the tail vascular plexus (a). The red channel image was omitted in panel b to highlight the newly formed microvascular network.

well as early dissemination of malignant cells, invasiveness and metastasis [67].

As described above, the first studies about extravasation dynamics of tumor cells in zebrafish were performed by Stoletov et al. in juvenile animals [25]. More recently, the same group utilized 48 hpf zebrafish embryos to visualize the extravasation of stable fluorescent highly metastatic human tumor cells injected into the pericardium. By 3–5 h, tumor cells enter the blood circulation and arrest in small vessels in the head and tail regions. Then, extravasation occurs and involves the modulation of tumor cell adhesion to the endothelium without damage of vascular bed during the extravasation process and the intravascular migration of tumor cells along the luminal surface of blood vessels independently from blood flow direction in a β1-integrin-dependent manner [69].

In our laboratory, we have followed the fate of highly metastatic murine melanoma B16-BL6 cells when injected directly into the embryonic blood circulation in the ventral region of the duct of Cuvier of transgenic tg(fli1:EGFP)^{y1} [70] zebrafish embryos at 48 hpf. To this purpose, cells were stably transfected with DsRed fluorescent protein, thus generating DsRed-B16-BL6 cells. In agreement with previous observations [55,69], injected cells (80–100 cells/embryo) disseminate throughout the whole embryo body within the blood circulation (Fig. 1B). One day after injection, cells extravasate in different anatomical sites, mainly in the tail region (Fig. 1C). This is followed during the

Table 1
Identification of anti-angiogenic agents by tumor cell engrafting in zebrafish embryo.

Tumor graft	Anti-angiogenic agent	References
Murine tumorigenic endothelial FGF2-T-MAE cells	FGF receptor-1 inhibitor SU5402; VEGF receptor-2 inhibitor SU5416; FGF2 inhibitor Ac-ARPCA-NH_2 pentapeptide	[41,75]
Murine melanoma B16/F10 cells	VEGF receptor-2 TK inhibitors SU5416 and SKLB1002	[55,76]
Human non-small cell lung carcinoma H1299 cells	VEGF receptor TK inhibitor PTK787/ZK222584	[52]
Human glioma U87MG cells	γ-Secretase inhibitors DAPT and compound E	[77]
Murine melanoma B16/F10 cells and human breast cancer MDA-MB-231 cells	PI3K inhibitor LY294002-loaded nanoparticles	[78]
Human sarcoma HT1080 cells	Thalidomide-loaded carbon nanotubes	[31]
Cisplatin resistant-human ovarian carcinoma OVCA 433 cells	$MEK_{1/2}$ inhibitor UO126	[79]
Human chronic myelogenous leukemia K562 cells	BCR-Abl1 inhibitor imatinib	[80,81]
Human acute promyelocytic leukemia NB-4 cells	PML-RARA inhibitor all-*trans* retinoic acid	[80]
Human acute T cell leukemia Jurkat cells	Cyclophosphamide and mafosfamide	[81]

next 3–4 days by the neovascularization (Fig. 1D) and growth (Fig. 2) of tail micrometastases. Similar results were obtained by He et al. after injection of different tumorigenic human and murine cell lines [47]. Again, vascular remodeling and angiogenesis play a pivotal role in tumor growth and invasion in this model. Of note, myeloid cells appear to control tumor invasion in a VEGF receptor-dependent manner by conditioning the collagen matrix and forming a metastatic niche in the caudal haematopoietic tissue of embryo tail [47].

3.3. *Advantages and disadvantages of the tumor xenotransplantation zebrafish embryo assays*

When compared to other in vivo tumor assays, zebrafish embryo/tumor xenograft models present several advantages. i) They allow the in vivo delivery of a very limited number of cells, mimicking the initial stages of tumor angiogenesis and metastasis. ii) Labeled tumor cells (e.g. GFP-transduced, DsRed-transduced or fluorescent dye-loaded cells) can be easily visualized following injection in transgenic zebrafish lines that exhibit fluorescent vasculature like Tg(fli1:eGFP) animals. Thus, analysis of the spatial/temporal relationship among tumor cells and newly formed blood vessels can be performed by non-invasive high-resolution imaging. iii) Several techniques can be applied within the constraints of paraffin or gelatin embedding, including histochemistry and immunohistochemistry. Electron microscopy can also be used in combination with light microscopy. Moreover, whole mount in situ hybridization and reverse transcriptase-polymerase chain reaction analysis with species-specific probes allow the study of gene expression by grafted tumor cells and by the host under different experimental conditions [41].

Zebrafish embryo/tumor xenograft models may represent a short-term assay suitable for the identification of novel tumor angiogenesis and/or metastasis inhibitors. Together with the rapid response, a large number of embryos can be injected and maintained in 96 well-plates, thus allowing systemic in vivo treatment of the animals with minimal amounts of compound. Therefore, dose–response experiments can be easily performed and numerous compounds can be tested in an effective manner. Relevant to this point, automated bio-imaging assays, microinjection robotics and microfluidic systems have been developed in zebrafish embryo that are applicable for angiogenesis and cancer cell dissemination studies and for the screening of anticancer drugs [29,71–74].

However, the metabolic fate of the drug (either in terms of its activation or inactivation) may differ in zebrafish embryo in respect to mammalian species. Also, zebrafish embryos are maintained at 28 °C. This may not represent an optimal temperature for mammalian cell growth and metabolism, even though we have observed mitotic figures with no sign of apoptosis in grafted tumors throughout the whole experimental period [41]. In this respect, the possibility to raise the incubation temperature up to 35 °C with no apparent gross effects on zebrafish development has been reported [36].

Species-specific microenvironmental differences may affect the behavior of grafted mammalian tumor cells and the absence of certain organs in fishes (including lung, mammary gland, and prostate) precludes the possibility to perform orthotopic transplantation experiments and to investigate tissue-specific mechanisms of tumor cell homing and colonization in these organs. To this respect, a large supply of zebrafish cancer cell lines, as well as of antibodies to zebrafish proteins, is sorely needed.

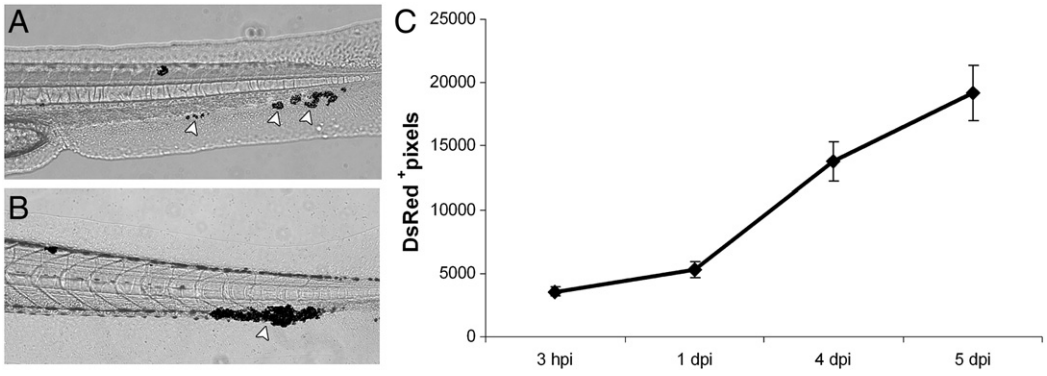

Fig. 2. Growth of tumor micrometastases in zebrafish embryo. Labelled murine melanoma DsRed-B16-BL6 cells were injected in the blood stream of transgenic tg(fli1:EGFP)^{y1} zebrafish embryos (80–100 cells/embryo) at 48 hpf. At different times after injection the embryos were analyzed by fluorescence microscopy in the tail region. DsRed-B16-BL6 cells arrested in the tail vascular plexus were photographed in the same embryo at 3 hpi (A) and 5 dpi (B) and images were processed to highlight tumor cells (in black). Note how the few tumor cells arrested at 3 dpi have formed an evident micrometastasis at 5 dpi (arrowheads). The relative rate of growth of tail micrometasases was quantified by computerized image analysis (C). Data are the mean ± SEM. of 29 embryos.

In summary, with its own advantages and disadvantages, mammalian tumor cell grafting in zebrafish embryos represents a novel tool for investigating the tumor/endothelial cell cross-talk during tumor growth, neovascularization and metastatic dissemination exploitable for gene targeting and drug discovery in cancer.

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