Antiangiogenic Cancer Drug Using the Zebrafish Model

Massimo M. Santoro

Abstract—The process of de novo vessel formation, called angiogenesis, is essential for tumor progression and spreading. Targeting of molecular pathways involved in such tumor angiogenetic processes by using specific drugs or inhibitors is important for developing new anticancer therapies. Drug discovery remains to be the main focus for biomedical research and represents the essence of antiangiogenesis cancer research. To pursue these molecular and pharmacological goals, researchers need to use animal models that facilitate the elucidation of tumor angiogenesis mechanisms and the testing of antiangiogenic therapies. The past few years have seen the zebrafish system emerge as a valid model organism to study developmental angiogenesis and, more recently, as an alternative vertebrate model for cancer research. In this review, we will discuss why the zebrafish model system has the advantage of being a vertebrate model equipped with easy and powerful transgenesis as well as imaging tools to investigate not only physiological angiogenesis but also tumor angiogenesis. We will also highlight the potential of zebrafish for identifying antitumor angiogenesis drugs to block tumor development and progression. We foresee the zebrafish model as an important system that can possibly complement well-established mouse models in cancer research to generate novel insights into the molecular mechanism of the tumor angiogenesis. (Arterioscler Thromb Vasc Biol. 2014;34:1846-1853.)

Key Words: angiogenesis inhibitors ■ drug screening ■ fluorescent imaging ■ pathological neovascularization ■ zebrafish

Tumor Angiogenesis

A tumor consists of a rapidly dividing and growing population of cells that have lost their ability to divide in a controlled fashion. Once a tumor lesion exceeds a few millimeters in diameter, hypoxia and nutrient deprivation trigger an angiogenic switch that allows the tumor to receive oxygen and nutrients from the bloodstream to sustain its growth.1 To achieve this, tumor cells exploit their microenvironment by releasing cytokines and growth factors to activate normal, quiescent cells around them and initiate a cascade of events. These events include the formation of new blood vessels, a process called tumor angiogenesis, as well as a recruitment of new tumor-associated cells such as macrophages and fibroblasts.2-4 Both tumor cells and tumor-associated cells contribute to the vascularization by activating and recruiting endothelial cells within blood vessels that are close to the tumor microenvironment.5,6 Traditional antiangiogenic strategies attempt to block new tumor blood vessel formation and destroy existing vessels by starving cancer cells from its nutrients.7,8 As a consequence, various antiangiogenic agents (eg, vascular endothelial growth factor blockers) that were originally developed to block tumor angiogenesis have been tested and used in clinical trials.9 However, it is now clear that the success of these traditional antiangiogenic strategies is restricted by their insufficiency within cancer cells to the inhibitors. This happens because tumor vessels themselves are abnormal, almost all aspects of their structure and function are extremely adaptable, making it nearly impossible to know how they will respond to treatments once they begin to develop.10 Therefore, preclinical and initial clinical evidence have both revealed that the normalization of the vascular abnormalities is an emerging, feasible, and an effective complementary therapeutic approach for cancer.11 It has been demonstrated that new mechanisms of tumor vessel normalization exist and that they can restore the normal basic features of endothelial cells within a tumor mass (eg, reducing metastatic dissemination from oxygen-enriched tumors and improving the response to conventional anticancer therapies).11,12 To design drugs that can be used to block such malignant processes, recent advanced strategies were aimed to better understand tumor vessels formation and normalization.13

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Looking for Preclinical Models of Antitumor Angiogenesis Drug Testing

To design new therapeutic strategies and to test the efficacy of antiangiogenic cancer drugs, researchers and clinicians need models in which these malignant angiogenic events can be
replicated and then studied at genetic, biochemical, molecular, and cellular levels. To date, the majority of the antiangiogenesis drug studies have been performed using endothelial cells isolated from either capillaries or large vessels.\textsuperscript{14,15} These in vitro or ex vivo techniques allow researchers to analyze the basic features of the angiogenic cascade that include endothelial cell proliferation, sprouting, migration, and differentiation. Although the initial phases of drug assessment by in vitro assays are important to determine drug–target affinity, they do not evaluate the actual range of drug interactions or the drug effects in the context of an intact tissue or an intact organism. Endothelial cell culture models can be useful to validate molecular and pharmacological targets as well as to screen for specificity and potency of potential drug candidates. However, this model lacks the most critical features that represent the interaction between vessel cells and the tumor mass as well as cancer-associated cells such as M2 macrophages, mural cells, and cancer-associated fibroblasts. Taking this into consideration, it is not surprising that many drug and inhibitory compounds (eg, blocking Ab) that were effective in the in vitro models of antiangiogenesis have produced disappointing results once they were tested in clinical trials.

To develop and test effective antitumor angiogenesis therapies, researchers should consider in vivo models of angiogenesis in which the different components of the tumor microenvironment, such as the tumor-host interface, are maintained and can be taken into account.\textsuperscript{16,17} Mammalian models are the most representative of human pathophysiology and, in fact, extensive tests in mammalian models (eg, rodents) are reasonable for testing single drugs in the late phase of their clinical trial. However, financial and time-consuming challenges make them unrealistic for large-scale drug screenings.\textsuperscript{18,19} Thus, it is necessary to use an alternative cost-effective vertebrate model with a sufficient complexity to fully resemble the in vivo mode of action of a drug. In scientific laboratories, simple vertebrate models can help to understand the early stages of drug development, including efficacy and specificity, but also off-target effects and toxicity of such drugs. The Xenopus, the chick, and the zebrafish are the most commonly found vertebrate models in scientific research. The Xenopus embryo has been used as a robust model for identifying cellular and molecular mechanisms involved in angiogenesis.\textsuperscript{20} An alternative vertebrate model, such as the chick embryo, has also been widely used in the past for such studies.\textsuperscript{21} The chorioallantoic membrane technique has been used as an experimental in vivo assay for studying both angiogenesis and antiangiogenesis responses. Several articles have been reported about the use of chorioallantoic membrane in response to tissues, cells or soluble factors, and drugs in the study of tumor angiogenesis.\textsuperscript{22} With respect to other vertebrate models, the zebrafish, \textit{Danio rerio}, is a more solid and powerful system to study both physiological and pathological angiogenesis. The zebrafish has been successfully used to uncover the correlations between tumor angiogenesis, inflammation, and metastasis.\textsuperscript{23,24} More recently, the integration of zebrafish transgenic technology with human cancer biology have helped in the development of zebrafish cancer models that target specific organs or cell types (eg, macrophages or fibroblast) within the tumors.\textsuperscript{25–27} In this review, we provide evidence that the zebrafish system is the best nonmammalian model for the rapid development of therapeutic drug agents aimed to block tumor angiogenesis in human patients. We also propose the zebrafish as an economical and physiologically relevant model system for the screening of antitumor angiogenesis candidate drugs.

**Angiogenesis and Imaging: A Lesson From the Zebrafish Model**

The zebrafish, \textit{Danio rerio}, system has emerged in the past few years as an ideal vertebrate model organism in which scientists can study a wide variety of biological processes.\textsuperscript{24,28a} Together with its small size and low cost maintenance, the advantages of the zebrafish model system include (1) high fecundity rates, each female capable of laying 200 to 300 eggs per week, (2) external fertilization that permits easy manipulation of embryos ex utero, and (3) rapid development because organogenesis is complete in few days after fertilization. These attributes have led to the emergence of the zebrafish as an important embryological model. The zebrafish system became popular when researchers started to show how feasible this model organism is to study vascular development and maturation.\textsuperscript{29–31} Vascular studies in zebrafish have yielded new insights into the anatomy of vascular development, including the dynamics of growing blood vessels (eg, vasculogenesis and angiogenesis) and the specification of arteries, veins, and mural cells.\textsuperscript{32,33} More recently, the benefit of using the zebrafish as a model to study hematopoiesis has also been highlighted.\textsuperscript{34} The advantage of studying angiogenesis in zebrafish resides in its optical clarity and easy genetic manipulation.\textsuperscript{35–37} These unique properties allow for the rapid generation of fluorescent (eg, green fluorescent protein based) transgenic lines that can be coupled with different optical imaging techniques to follow formation, wiring, and shaping of blood vessels in vivo and in real time\textsuperscript{38} (Movie I in the online-only Data Supplement). Each of these attributes (optical transparency, easy developmental manipulation, and the growing number of genetic tools available) make zebrafish an exceptional model system to study not only developmental angiogenesis but also the de novo angiogenesis associated with pathological conditions such as tumor vascularization.\textsuperscript{39,40}

**Cancer Models of Tumor Angiogenesis in Zebrafish**

The zebrafish system is now viewed as a promising model to study the many aspects of human cancer.\textsuperscript{41} It has been shown that cancer progression in these animals recapitulates many aspects of the human disease, both genetically and histologically, offering a unique opportunity for exploiting the genetic and chemical modifiers of cancer.\textsuperscript{25–42} The importance of zebrafish for cancer studies has been fueled by the establishment of both heritable and transplantable tumor models.\textsuperscript{43} Researchers can use 2 different ways to generate cancer models: the genetic cancer model and the transplantable model (Table 1). The first one is based on the manipulation of genes within fish tissues to generate tumor masses or cancer. The genetic manipulations aim to inactivate a tumor suppressor gene or, alternatively, to
overexpress an oncogene. Specifically, inactivation of zebrafish tumor-suppressor gene (eg, \textit{p53}, \textit{apc}, and \textit{pten}) has been achieved using targeting induced local lesions in genomes and forward genetic screens. On the other hand, oncogene expression has been achieved by generating transgenic zebrafish lines expressing certain oncogenes (eg, \textit{kras}, \textit{NRAS}, and \textit{MYCN}) under specific promoters. The second way to study cancer with the zebrafish is based on grafting (transplant) tumor cells within zebrafish tissues or organs. The hetero (xeno) graft model is characterized by transplantation of tumor cells of human or mouse origins. Instead, the homo (allo) graft model is characterized by transplantation of tumor zebrafish cells. In both cases, these cancer cells grow within the site of injection, generating a tumor that uses the host environment (eg, blood vessels) to grow, expand, and even invasion.

In the last years, it has been shown that these zebrafish cancer models represent an optimal system to study tumor-induced angiogenesis in vivo. In most of these models, tumor cells can promote neoangiogenesis from existing vessels, thereby validating both heritable and transplanted zebrafish tumor models to study tumor angiogenesis and even invasion processes in entire vertebrate animals\cite{26,44-49}. These cancer models when combined with specific other zebrafish properties, such as optical transparent adult animals (eg, \textit{Casper}), forward genetic screens (eg, targeting induced local lesions in genomes), reverse genetics (eg, transcription activator-like effector nucleases or clustered regularly interspaced short palindromic repeats(Cas9)), site-specific recombinase technology (eg, Cre-Lox), high efficient transgenesis (eg, Tol2 transposon system), advanced optical imaging (eg, confocal and light sheet microscopy), and ease of microinjection and tissue transplantation (eg, tumor xenograft or cancer stem cell engraftment), result as an ideal model to study tumor angiogenesis.

<table>
<thead>
<tr>
<th>Table 1. Zebrafish Cancer Models</th>
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<tbody>
<tr>
<td><strong>Genetic cancer models</strong></td>
</tr>
<tr>
<td>1.1 Gene (tumor suppressor) inactivation</td>
</tr>
<tr>
<td>\textit{tp53}</td>
</tr>
<tr>
<td>\textit{apc}</td>
</tr>
<tr>
<td>\textit{pten}</td>
</tr>
<tr>
<td>1.2 Gene (oncogene) expression</td>
</tr>
<tr>
<td>\textit{MYCN}</td>
</tr>
<tr>
<td>\textit{BRAP}^{\textit{p300}} (mitfa promoter)</td>
</tr>
<tr>
<td>\textit{NRAS}^{\textit{v61}} (mitfa promoter)</td>
</tr>
<tr>
<td>\textit{HRAS}^{\textit{g12v}} (kita promoter)</td>
</tr>
<tr>
<td>\textit{TEL-AML1} (\textit{g-actin} promoter)</td>
</tr>
<tr>
<td>\textit{TEL-AML1} (\textit{kif6} promoter)</td>
</tr>
<tr>
<td>\textit{c-Myc} (\textit{rag2} promoter)</td>
</tr>
<tr>
<td>\textit{KRAS}^{\textit{G12k}} (\textit{Ptf1a} locus)</td>
</tr>
<tr>
<td>\textit{kras}^{\textit{V12}} (\textit{fabp10} promoter)</td>
</tr>
<tr>
<td>\textit{KRAS}^{\textit{G12e}} (\textit{rag2} promoter)</td>
</tr>
</tbody>
</table>

| **Transplant models**           |          |            |
| 2.1 Hetero (xeno) graft         |          |            |
| Murine B16-BL16 cells           | Melanoma cells | 44,63,79     |
| Murine FF2-T-MAE cells          | Tumorigenic endothelial cells | 63          |
| Murine CL-13 cells              | Lung adenocarcinoma | 39          |
| Human MDA-MB-435 or 231 cells   | Breast carcinoma | 63,79-81     |
| Human PC3 cells                 | Prostate cancer | 82          |
| Human HT1080 cells              | Fibrosarcoma | 79          |
| Human OVCA cells                | Ovarian carcinoma | 80,83       |
| Human PaTu-T and Panc-1 cells   | Pancreatic cancer | 84          |
| Human A2780 cells               | Ovarian carcinoma | 46          |
| Human U87 cells                 | Glioblastoma | 49          |
| 2.2 Homo (allo) graft           |          |            |
| Embryonic rhabdomyosarcoma cells | Rhabdomyosarcoma | 78          |
| Melanoma cells                  | Melanoma | 72          |
| T-ALL cells                     | Leukemia | 76          |
Differences exist between these 2 ways of simulating tumor growth in vivo regarding their capacity to be translated into clinical successful antitumor angiogenesis treatments. Genetic cancer models are characterized by gene-base tumor cell transformation and slow-growing tumor mass that have the advantage to recapitulate the spontaneously arising and slowly evolving cancers of human patients. However, it is also possible that spontaneous tumor models in genetically engineered fish do not also recapitulate various aspects of the human diseases (differences in malignancies, vascularization, stromal microenvironment, noncell autonomous effect may lead to different responses). Zebrafish transplant models recapitulate rapidly growing primary tumors. Advantages of the zebrafish transplant model would include the speed of analysis for tumor angiogenesis and the possibility to perform whole-mount in situ gene expression analyses in heterologous tumor cells comparing it with the tumor-induced gene expression in the surrounding host environment. The transplant of cancer cells can be done both in embryo–larvae stages and in juvenile–adult stages. The major advantage of the transplantation in early embryos is the fact that their immune system is still immature, and this permits (human) cancer cells to engraft without rejection. The advantages of using late transplant models will include easy interpretation of tumor-induced vascular effects because most of the organs in the juvenile fish, including the vasculature, are fully formed including smooth muscle cells and pericytes. On the other hand, a limitation for the xenotransplant model is the requirement of chemical suppression of the host’s immune function for successful grafting of the human cancer cells (that would be avoided in case of homografts).

The generation and use of these cancer models in zebrafish thus provide a unique insight into the mechanisms underlying tumor angiogenesis and, more importantly, a potential platform for new drug discovery.

Drug Testing and Screening in Zebrafish

Because of its permeability to small molecules, zebrafish can be used for testing and screening of drugs affecting different biological processes. Single and multiple compounds tests can be easily and successfully achieved on both zebrafish embryos and adults. The reason for this resides in the fact that (1) a high quantity of zebrafish embryos (∼5000) can be obtained synchronously, and (2) the small size of the embryos allows them to fit in a 384-well plate and thus allows high-throughput analyses. In this way, libraries of thousands of compounds can be screened for their effects in a reasonable time frame (eg, weeks). Progresses for drug screening in zebrafish embryos are also subjected to automatic readout for phenotypic effects. An automated high-throughput platform for in vivo chemical screenings on zebrafish embryos has been developed aiming for the highest possible throughput and minimization of human error. It includes an automated method for embryo collection and preparation, compound delivery, incubation, imaging, and analysis of results.

Another advantage of zebrafish embryos for drug testing and screening is their rapid development; this allows the assays to be performed in a relatively short time (eg, days). Therefore, the dissection of the phenotype of interest and the evaluation of possible side effects and toxicity can be done effortlessly. An idyllic antitumor drug is a molecular one that only interacts with a single specific target and exerts the desired therapeutic effect with high affinity and specificity. Unfortunately, such ideal drugs are a great challenge to develop. Despite how cautiously a drug–target interaction has been measured by in vitro assays and bioinformatics tools, many drug candidates display off-target effects in complex organisms, such as the mouse. This off-target effect needs to be balanced against the potential therapeutic benefit for the patient. As a matter of fact, zebrafish drug screening assays are becoming a reliable tool and are routinely used in preclinical safety evaluations because their ability to accurately predict toxicity in mammals can be firmly established. The zebrafish system is emerging as a valuable model organism for drug discovery processes that include target identification, disease modeling, lead discovery, and toxicology.

Two important areas of drug screening in which zebrafish model proves to be more advantageous are structure–activity relationship and high-content screening. In the structure–activity relationship process of drug discovery, once a candidate compound is selected for its biological activity, it could be modified to improve pharmaceutical features or reduce side effects. Structural analogs of the parent compound could be prepared and tested in for screening directly in zebrafish.

**Table 2. Antitumor Angiogenic Drugs Identified by Xenograft Assays in Zebrafish Embryos**

<table>
<thead>
<tr>
<th>Antiangiogenic Drugs</th>
<th>Transplanted Cells</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>SU5416 (VEGFR-2 kinase inhibitors)</td>
<td>Melanoma B16/F10 cells</td>
<td>48</td>
</tr>
<tr>
<td>SKLB1002 (VEGFR-2 kinase inhibitors)</td>
<td>Melanoma B16/F10 cells</td>
<td>85</td>
</tr>
<tr>
<td>PTK787/ZK222584 (VEGFR kinase inhibitor)</td>
<td>Non–small-cell lung carcinoma H1299 cells</td>
<td>39</td>
</tr>
<tr>
<td>LY294002 (PI3K inhibitor)</td>
<td>Breast cancer MDA-MB-231 cells</td>
<td>81</td>
</tr>
<tr>
<td>SU5402 (FGFR-1 inhibitor)</td>
<td>Tumorigenic endothelial cells (FGF2-T-MAE)</td>
<td>63</td>
</tr>
<tr>
<td>U0126 (MEK1/2 inhibitor)</td>
<td>Human ovarian carcinoma OVCA 433 cells</td>
<td>83</td>
</tr>
<tr>
<td>DAPT (γ-Secretase notch inhibitor)</td>
<td>Human glioma U87MG cells</td>
<td>86</td>
</tr>
<tr>
<td>Imatinib (BCR-Abi inhibitor)</td>
<td>Human chronic myelogenous leukemia K562 cells</td>
<td>87</td>
</tr>
<tr>
<td>All-trans retinoic acid (PML-RARA inhibitor)</td>
<td>Human acute promyelocytic leukemia NB-4 cells</td>
<td>88</td>
</tr>
</tbody>
</table>

BCR indicates breakpoint cluster region; FGFR, fibroblast growth factor receptor; MEK, mitogen-activated protein kinase kinase; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PML-RARA, promyelocytic leukemia-retinoic acid receptor alpha; and VEGFR, vascular endothelial growth factor receptor.
By doing structure–activity relationship studies in zebrafish, structures can be identified that improve potency (eg, binding affinity) without increased toxicity or loss of in vivo efficacy.\textsuperscript{58} Although cell-based assays exist, rapid and cost-efficient HCS assays within intact organisms are needed to support prioritization for developmental antiangiogenesis testing in zebrafish. The development of automated microscopy platforms has enabled large-scale observation of biological processes, thereby complementing genome-scale biochemical techniques. To this end, using transgenic zebrafish (kdrl1:gfp) that stably express green fluorescent protein within blood vessels, we can develop and optimize 96-well-based HCS assays that enable us to rapidly screen and identify chemicals impacting tumor but not developmental angiogenesis at nonteratogenic concentrations. We recently demonstrate a toolset for automated intelligent HCS of heart rate in zebrafish embryos at tumor but not developmental angiogenesis for developmental antiangiogenesis testing in zebrafish.\textsuperscript{61,62} Recently, it has become realistic to generate a novel tumor angiogenesis inhibitor by using the different zebrafish cancer models. On this line, we used the zebrafish transplantable cancer model to identify new antitumor angiogenesis drugs by performing small-scale chemical screening on xenografted zebrafish embryos. The original concept is to inject tumorigenic cells in the zebrafish embryos and treat those embryos with a library of chemicals and then select those that specifically block tumor (but not developmental) angiogenesis (eg, de novo blood vessels around the tumor mass; Figure). It would be interesting to use gene-based cancer models for resembling the spontaneously arising and slow growth of tumors. The presence of highly vascularized genetic models in zebrafish (eg, melanomas, pancreatic and liver models) is becoming undeniably interesting because it represents the starting point for high-throughput screening of antiangiogenic drugs in genetic-based model of tumor angiogenesis in zebrafish.\textsuperscript{51,63} Recently, it has become realistic to generate a novel zebrafish model for studying cancer stem cell invasion in vivo, opening for the possibility to screen for drugs that block tumor invasion and metastatization.

Antitumor Angiogenesis Drug Screening in the Zebrafish Model

Because of its permeability to small molecules and easy blood vessel imaging, the zebrafish has been extensively used as optimal model to study antiangiogenic compounds and screening for new drugs, with quantitative and automated readout assays\textsuperscript{59,60} (Table 2).

The next challenge is the identification of specific tumor angiogenesis inhibitors by using the different zebrafish cancer models. On this line, we used the zebrafish transplantable cancer model to identify new antitumor angiogenesis drugs by performing small-scale chemical screening on xenografted zebrafish embryos. The original concept is to inject tumorigenic cells in the zebrafish embryos and treat those embryos with a library of chemicals and then select those that specifically block tumor (but not developmental) angiogenesis (eg, de novo blood vessels around the tumor mass; Figure). It would be interesting to use gene-based cancer models for resembling the spontaneously arising and slow growth of tumors. The presence of highly vascularized genetic models in zebrafish (eg, melanomas, pancreatic and liver models) is becoming undeniably interesting because it represents the starting point for high-throughput screening of antiangiogenic drugs in genetic-based model of tumor angiogenesis in zebrafish.\textsuperscript{51,63} Recently, it has become realistic to generate a novel zebrafish model for studying cancer stem cell invasion in vivo, opening for the possibility to screen for drugs that block tumor invasion and metastatization.

Fact or Fiction: Shortcomings and Limitations of the Zebrafish Model in Studying Cancer and Tumor Angiogenesis

Zebrafish exhibit unique characteristics, including ease of maintenance, drug administration, a short reproductive cycle, and physical transparency, that permit visual assessment of developing cells and organs. The advantages of zebrafish bioassays are that they are cheaper and faster than those in mammalian models and are suitable for cost-effective large-scale drug screening.\textsuperscript{51} The recent establishment of spontaneous cancer models in fish is becoming an interesting alternative to xenograft assays that would overcome some of its limitations.\textsuperscript{63} Although it has recently become a promising model system in cancer biology, the zebrafish model shows some limitations and drawbacks to study several aspects of cancer progression, such as tumor angiogenesis. This is true if compared to established mammalian model such as the rodents.\textsuperscript{52,64} First, there are large differences in organ/body size between zebrafish and mammals as well as the absence of critical vascularized organs that could interfere with the delivery and mode of action of antiangiogenic drugs (eg, lung and skin). As a consequence, there is a different biological...
microenvironment and niche structure that might interfere with drug delivery, metabolism, and efficiency. The idea to study vessel abnormalization of tumor models in zebrafish would be an exciting opportunity not yet investigated. The idea of studying new molecular pathways associated with vessel normalization of the tumor vasculature is interesting, considering the easy genetic amenability of the zebrafish model to perform molecular and genetic studies associated with advance imaging technique such as electron microscopy.

Taking advantage of optical clarity and availability of Tg lines, marking different component of the hematopoietic and immune system in zebrafish, would be interesting to analyze the contribution of tumor-associated immune cells (eg, macrophages) and fibroblasts in tumor angiogenesis models both in zebrafish embryos and adults.

Although fast and time-efficient cancer models are rapidly growing, these primary tumors might not entirely recapitulate the spontaneously arising and slowly evolving invasiveness of cancers observed in humans. As a matter of fact, the zebrafish model is also a cost-effective system for better understanding the mechanisms used by tumor cells for invasion and spread, opening to the idea to use it for the identification and testing of novel antitumor invasion/metastasis agents. As a consequence, the antiangiogenic approaches designed in zebrafish might consider these new features in the near future.

Considering the transplant cells of different origins in zebrafish embryos, the role of stromal changes caused by chronic inflammation and activation of macrophages, neutrophils, and cancer-associated fibroblasts may contribute to the initiation of cancer and can be investigated in the zebrafish model. It is worthwhile to consider the zebrafish model as a saleable approach to study the mechanisms underlying cancer stem cells propagation as well as for high-throughput screening of novel anticancer stem cell drugs.

Overall, future applications of the zebrafish might provide strength and motivation for implementing the use of the zebrafish system as a preclinical model to study human cancer and develop new therapies for treatment.
5. Vessels as targets in cancer.
6. Ubiad1 is an antioxidant enzyme that regulates eNOS activity by CoQ10.


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