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Utilizing zebrafish to identify anti-(lymph)angiogenic compounds for cancer treatment: promise and future challenges

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Abstract

Cancer metastasis which predominantly occurs through blood and lymphatic vessels, is the leading cause of death in cancer patients. Consequently, several anti-angiogenic agents have been approved as therapeutic agents for human cancers such as metastatic renal cell carcinoma. Also, anti-lymphangiogenic drugs such as monoclonal antibodies VGX-100 and IMC-3C5 have undergone phase I clinical trials for advanced and metastatic solid tumors. Although anti-tumor-associated angiogenesis has proven to be a promising therapeutic strategy for human cancers, this approach is fraught with toxicities and development of drug resistance. This emphasizes the need for alternative anti-(lymph)angiogenic drugs. The use of zebrafish has become accepted as an established model for high throughput screening, vascular biology and cancer research. Importantly, various zebrafish transgenic lines have now been generated that can readily discriminate different vascular compartments. This now enables detailed *in vivo* studies that are relevant to both human physiological and tumor (lymph)angiogenesis to be conducted in zebrafish. This review highlights recent advancements in the zebrafish anti-vascular screening platform and showcases promising new anti-(lymph)angiogenic compounds that have been derived from this model. In addition, this review discusses the promises and challenges of the zebrafish model in the context of anti-(lymph)angiogenic compound discovery for cancer treatment.

Abbreviations used

ABCB: ATP-binding cassette, sub family B (MDR/TAP)

BMP: Bone morphogenetic protein

CCBE1*:* Collagen and calcium binding EGF domains 1

DMSO: Dimethyl sulfoxide

DPF: Days post-fertilization

DSRED2: *Discosoma* sp. Red fluorescent protein 2

FDA: Food and Drug Administration

FGF2: Fibroblast growth factor 2

FLI1a: Friend leukemia integration 1 transcription factor proto-oncogene, E26 transformation-specific transcription factor a

HPF: Hours post-fertilization

KDRL: Kinase insert domain receptor like

LYVE1B: Lymphatic vessel endothelial hyaluronic receptor 1b

NEGFP: Nuclear enhanced green fluorescent protein

PDGF: Platelet-derived growth factor

PKD1A: Polycystic kidney disease 1a

PHKG1: Phosphorylase kinase subunit G1

PROX1A: Prospero homeobox 1a

RFP: Red fluorescent protein

VEGF: Vascular endothelial growth factor

VEGFR: VEGF receptor

Introduction

Metastasis remains the leading cause of death in cancer patients, accounting for ~90% of human cancer deaths [105]. Apart from supplying nutrients and oxygen to rapidly

proliferating cancer cells and relieving interstitial pressure within the tumor microenvironment, blood and lymphatic vessels play key roles in disease progression by providing in essence, conduits for cancer cell dissemination [41,133]. Of note, tumor cells combined with tumor-associated macrophages and fibroblasts, in turn are able to promote both angiogenesis and lymphangiogenesis, formation of new blood and lymphatic vessels, respectively from pre-existing vessels primarily by the upregulation of key pro-(lymph)angiogenic growth factors such as VEGF-A, VEGF-C, VEGF-D, FGF2 and PDGF-BB [47]. Notwithstanding, elevated (lymph)angiogenesis growth factors is now known to be associated with a higher incident of cancer metastasis and poor prognosis [120,132]. Although recent studies highlight alternative routes for cancer dissemination for example the nervous system [99], blood and lymphatic vessels remain the most well characterised pathway for cancer metastasis.

The intimate relationship between angiogenesis and cancer had led the late Dr Judah Folkman in 1971, to hypothesize that blood vessels were essential for cancer growth and that anti-angiogenic agents could be developed as therapeutic agents for cancer therapy [40]. Despite receiving initial criticisms, this concept has now been widely accepted by the scientific community and consequently, has yielded several clinically approved anti-angiogenic drugs as treatment options for several human cancers. These include the humanized monoclonal VEGF-A antibody, bevacizumab, VEGF-A binding recombinant fusion protein, Ziv-Aflibercept and multi-kinase inhibitors (sorafenib, sunitinib, pazopanib, axitinib, cabozantinib, regorafenib), designed to inhibit VEGFR activity for example, have all gained approval as treatment options for metastatic colorectal cancer, metastatic breast cancer, non-small cell lung cancer, advanced renal cell carcinoma, gastrointestinal stromal tumor, advanced hepatocellular carcinoma and advanced thyroid carcinoma (reviewed in [130]). Although anti-tumor-associated angiogenesis has proven to be a promising therapeutic strategy for treating cancer, these are nevertheless associated with various side effects such as hypertension, proteinuria, impaired wound healing, gastrointestinal perforation, haemorrhage, thrombosis, reversible posterior leukoencephalopathy, cardiac toxicity and endocrine dysfunction [35]. Furthermore, development of resistance to this form of therapy is another compounding factor, whereby tumors seek alternative pro-angiogenic factors outside of the most commonly targeted VEGF-A for instance, angiopoietins, interleukins, FGFs, PDGFs, and hypoxia inducible factor 1α (HIF-1α) [13,43].

As an alternative strategy, the correlation between lymphangiogenesis and cancer has stimulated interest for the clinical evaluation of anti-lymphangiogenic agents as therapeutic options for cancer treatment [33]. Although not all cancers spread via the lymphatic system (such as sarcoma [67,145]), anti-lymphangiogenic therapies may offer benefit to a sub-set of tumors such as melanomas [31], head and neck [10], breast [119] and prostate cancers [72], which are known to spread via the lymphatic system. Additionally, combinational formulation of anti-angiogenic/lymphangiogenic compounds have shown promise in improving efficacy over anti-angiogenic therapies alone for certain cancers [7]. Examples of anti-lymphangiogenic agents under clinical evaluation includes a class of humanized monoclonal antibodies, VGX-100 and IMC-3C5, that specifically targets VEGF-C and VEGFR-3, respectively [7,104]. The phase I clinical evaluation of VGX-100 on patients with advanced solid tumors either as monotherapy or in combination with bevacizumab showed that the therapy was safe and well tolerated in patients up to a maximal pre-planned dose of 30 mg/kg [37] (NCT01514123). Combinational therapy of VGX-100 and bevacizumab showed a modest antitumor response with a disease control rate of 12%. Similarly, the phase I clinical evaluation of IMC-3C5 on advanced solid tumors including colorectal cancer patients refractory to standard therapy has recently been completed and broadly demonstrating that while the schedule was well tolerated at a maximal pre-planned dose of 30 mg/kg, minimal benefit was noted [125] (NCT01288989). Although approved multi-kinase inhibitors such as sunitinib also target lymphangiogenesis [80], there are currently no clinically FDA-approved therapies specifically targeting tumor lymphangiogenesis for cancer treatment, emphasizing an urgent need to identify compounds with anti-(lymph)angiogenic properties with minimal side-effects for developing as novel anti-cancer therapies.

Since the identification of functional zebrafish blood and lymphatic vessels, the zebrafish model had contributed immensely to our current knowledge of vascular biology [84,129]. Armed with this knowledge, the zebrafish model now affords a powerful screening tool for identifying novel anti-(lymph)angiogenic drugs as it offers an *in vivo* system that is amenable to large scale small molecule screens, low cost of maintenance, rapid developmental speed and high fecundity [127]. Additionally, zebrafish embryos develop *ex utero* enabling drug delivery to be conducted through drug bathing. Further, the optical transparency of zebrafish embryos and the availability of transgenic lines that fluorescently

label the vasculature further enhances the utility of the zebrafish as an excellent anti-(lymph)angiogenic drug screening platform (Figure 1A and 1B).

Of noteworthy, ~70% of human protein-coding genes have a zebrafish orthologue [65]. This suggests that most molecular mechanisms pertinent to normal and disease processes are highly conserved in zebrafish and drugs and/or compounds found to interfere with these biological processes can be readily translated to the context of human physiopathogenesis. For example, 16, 16-dimethyl prostaglandin E2 (PROHEMA®), a chemical derivative of prostaglandin E2, is a drug that was identified from a zebrafish-based experiment and has been brought forward to phase II clinical trial as potential therapy to improve the success rate of haematopoietic stem cell transplants using umbilical cord blood (NCT01627314) [30,111]. In this review, we highlight emerging techniques and tools, for example transgenic lines that can be readily applied to anti-(lymph)angiogenic screens in zebrafish to identify hits that show promise as anti-cancer therapeutics. We also describe approaches to quantify key blood/lymphatic vessels used for anti-(lymph)angiogenic screens such as intersegmental vessels, subintestinal vessels, and the thoracic duct as well as our current understanding of the molecular bases for development of these vessels, broadly to help identify only the very compelling hits for further validation and characterization.

(Lymph)angiogenic vessel development and quantification in zebrafish

Blood vessel development in larval zebrafish has been extensively investigated, in particular the development of the intersegmental and subintestinal vessels, representing two most commonly used vessels for angiogenic quantification in zebrafish [69,70]. Briefly, in zebrafish, vascular sprouts that contribute to intersegmental vessel development emerge from the dorsal aorta at approximately 18 hpf. These sprouts than grow dorsally between the somites and once they reach the level of the dorsal neural tube, they start migrating horizontally both anteriorly and posteriorly to form the dorsal longitudinal arterial vessel. Vessels that grow between the somites are termed intersegmental vessels. Subintestinal vessels on the other hand, start developing later compared to intersegmental vessels at approximately 30 hpf and were initially thought to originate from the common cardinal vein [69]. However, recent studies using detailed time-lapse analysis broadly revealed that the subintestinal vessels originate from the posterior cardinal vein and involve various mechanisms of endothelial cell migration such as vascular sprouting, anastomoses, pruning

and remodeling [46,57,81,95,109]. Of note, key angiogenic pathways involving for example, VEGF-A/VEGFR-2, Notch and Semaphorin/PlexinD1 are now known to be required for intersegmental and subintestinal vessel development, making it an excellent site for anti-angiogenic drug screens [25,27,28,46,57,81,96,131,143,174]. It is important to note however, that angiogenic factors mediating intersegmental vessel development such as PDGFs and sonic hedgehog (SHH) are not required for subintestinal vessel development, while BMP which is not required for intersegmental vessel development is essential for subintestinal vessel development [19,46,53,92,156]. This highlights the differing molecular mechanisms between developmental and organ-specific angiogenesis but provides an opportunity for investigators to gain insights for anti-angiogenic hits identified from using these vessels as windows. Intersegmental and subintestinal vessel development are readily quantifiable using various methods as depicted in Figure 1C- 1H'. Briefly, intersegmental vessel development can be quantified by determining the proportion of fully developed and mature intersegmental vessels [18] or by calculating the average lengths of the intersegmental vessels [158,169] using 30-48 hpf embryos (Figure 1C-1F'). On the other hand, subintestinal vessel development can be quantified broadly by either analyzing neo-vessel (interconnecting vessels) formation within the subintestinal vessel basket [87], size of the subintestinal vessels [171] or by counting the number of vascular compartments within the subintestinal vessels [46], using embryos at various developmental stages ranging from 55-80 hpf (Figure 1G-1H').

Unlike blood vessels, lymphatic counterparts in zebrafish have only recently been characterized and the thoracic duct is now regarded as a common site for lymphatic quantification. In this context, orthologues of mammalian lymphangiogenic genes for example, *vegfc* [86], *vegfr3* [62] and *neuropilin 2a (nrp2a)* [58], are now known to be required for zebrafish thoracic duct development. Furthermore, detailed analysis of the zebrafish thoracic duct has led to the identification of several novel genes that regulate both zebrafish and mammalian lymphangiogenesis, for example *pkd1a* [29], ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 3a (*arap3a*) [77], apelin (apln) and apelin receptor a (*aplnra*) [78], claudin-like protein 24 (*clp24*) [124] and *ccbe1* [61]. In particular, the discovery of *ccbe1* as a novel lymphatic gene has been one of the most important findings in this field, essentially as it was one of the first lymphatic genes with clinical implications identified using zebrafish. The role of *ccbe1* in vertebrate lymphangiogenesis was first discovered

when Hogan and colleagues characterized the *full of fluid* (*fof*) mutant zebrafish larvae identified from a forward genetic screen that isolated edemic mutants. Here, they showed that *fof* mutant larvae lacked lymphatic vessels and genetic mapping localized the mutation to the *ccbe1* gene. Subsequent zebrafish and murine-based experiments have revealed that CCBE1 is indispensable for vertebrate lymphangiogenesis as it indirectly activates VEGF-C via enhancing the A disintegrin and metalloproteinase with thrombospondin motifs-3 protease (ADAMTS3)-mediated cleavage and activation of VEGF-C [15,71,94] Importantly, mutations in the gene resulting in loss of function of CCBE1, is now associated with Hennekam syndrome, a rare inherited disorder manifesting in a defective lymphatic system and further reiterating that the molecular mechanisms involved in lymphatic sprouting in human and zebrafish are conserved [2].

In zebrafish, thoracic duct formation is initiated when secondary sprouts emerge from the posterior cardinal vein at approximately 1.5 dpf [86,163]. Notably, cells contributing to this sprouting arise from mesoderm-derived angioblasts located at the ventral side of the posterior cardinal vein and mediated by Wnt5b [109]. Furthermore, these cells are now known to divide asymmetrically to give rise to Prox1a-negative daughter cells that essentially remain in the vein, while the Prox1a-positive counterpart cells give rise to secondary sprout formation in a Vegfc-dependent manner [85]. Of interest, one half of the secondary sprouts containing Prox1a-positive cells migrate dorsally to form the parachordal lymphangioblasts along the horizontal myoseptum at approximately 48 hpf while the other half connect to the basal stalk of the primary intersegmental vessels to form the venous intersegmental vessels (the remaining intersegmental vessels are termed arterial intersegmental vessels) [16,44]. By 60 hpf, the parachordal lymphangioblasts migrate along the arterial intersegmental vessel both dorsally and ventrally to give rise to the dorsal longitudinal lymphatic vessel, intersegmental lymphatic vessels and the thoracic duct [61].

Pertinent for drug screening, thoracic duct development in zebrafish is readily quantifiable, by either counting the number of somites with thoracic duct or parachordal lymphangioblasts [6,21,29] or by counting the number of lymphatic endothelial cells within the zebrafish thoracic duct using for example, the *Tg(fli1a: nEGFP)*^{y1} transgenic line that has been genetically engineered to express nuclear fluorophores driven by the *fli1a* promoter within vascular endothelial cells (Figure 1I-1L') [85,123]. Quantification of parachordal lymphangioblast can be done using zebrafish embryos at approximately 55 hpf [29,77],

while for thoracic duct this measurement can be performed with post-5 dpf larvae. Absence of both the thoracic duct and parachordal lymphangioblasts indicates that the lymphatic sprouts failed to emerge from the posterior cardinal vein while absence of the thoracic duct but not parachordal lymphangioblasts shows that lymphatic endothelial cells migrated to the myoseptum to form parachordal lymphangioblasts but failed to migrate beyond that point. This scenario can occur when genes required for lymphatic endothelial cell migration or guidance for example, *pkd1a* [29] and chemokine (C-X-C motif) ligand 12 b (*cxcl12b*) [21] are not expressed.

Tools for anti-(lymph)angiogenic drug screens using zebrafish

Transgenic zebrafish lines

The classical preclinical testing for new drug targets entailed the use of established human cancer cell line models. However, the scope of this approach for generating clinical leads is often limited as results obtained from *in vitro* systems, often do not translate into whole organism, where drugs have to be absorbed through epithelial barriers, get exposed to metabolizing enzymes, and then find their respective targets [116]. In addition, the organ-specific toxicity of the clinical leads cannot be fully investigated using an *in vitro* system. Compounding this, is the high cost and time required for generating and maintaining mammalian mouse model systems, making them unsuitable for large scale drug screens [100]. Thus, zebrafish representing a simple vertebrate model, can serve as an early preclinical drug screening tool. To this end, a toolkit of experimental approaches that have been recently developed and optimized, now allow anti-(lymph)angiogenic screens to be readily conducted in zebrafish. Notwithstanding, earlier approaches relied on lower resolution microscopy, for example *in situ* hybridization [42,142] and alkaline phosphatase staining [25] which by requiring the use of fixed embryos, essentially made real-time live observations of vessels challenging. On the other hand, microangiography [154] and lymphangiography [86] are alternative methods for visualising vessels in a live zebrafish embryo but these methods are deemed too labour intensive to be incorporated into a drug screening platform.

One of the most prominent developments in the field of zebrafish (lymph)-angiogenesis without doubt, has been the generation of several flavours of transgenic lines that allows for high resolution real-time live imaging of vascular development [76]. An

added advantage with the use of these transgenic lines is that they afford means to perform rapid quantification of blood/lymphatic vessels in a live zebrafish without the need for additional manipulation. Examples of gene promoters used to generate transgenic zebrafish lines that are genetically engineered to fluorescently colour code blood/lymphatic vessels are listed in Table 1[1]. Key examples commonly used for quantifying both angiogenesis and lymphangiogenesis are transgenic lines that utilize the promoter of *fli1a*, *kdrl* or *lyve1b*. The *fli1a* gene is expressed in all vascular endothelial cells and hence transgenic lines of this gene permits visualization of both blood and lymphatic vessels [86,163]. On the other hand, *kdrl* gene is expressed predominantly in blood endothelial cells [66,73,137] and *lyve1b* gene is expressed in both primary veins and lymphatic endothelial cells [39,112], therefore allowing more specific quantification/research of blood or lymphatic vessel, respectively, to be conducted. Transgenic lines that express fluorophores with different colours can be taken advantage of to visualise different endothelial compartments within a single zebrafish. For example, compound transgenic lines such as the *Tg(fms-related tyrosine kinase 1 (flt1): RFP)^{hu5333};SAGFF27C;UAS:GFP* [16], the *Tg(lyve1b:DsRed2)^{nz101};Tg(kdrl:EGFP)^{s843}* [112], *Tg(fli1a:EGFP)^{y1};Tg(kdrl:EGFP)^{s843}* [62] and *Tg(prox1a:KalTA4)^{uq3bh};Tg(10×UAS:Venus);Tg(kdrl:mCherry)^{s916}* [85] enables venous, arterial, and lymphatic endothelial cells to be differentiated. These transgenic lines not only provide platforms for studying the interaction between these endothelial cell compartments [16,44] but are also useful tools for quantification of individual endothelial compartments. Importantly, combining these transgenic lines with high-end imaging capabilities such as confocal, multiphoton and light sheet microscopy, now allow real-time live visualization of vascular development at a resolution that is difficult to achieve in more complex murine models [85,109]. Collectively, availability of the aforementioned models and compatibility with real-time imaging provides an excellent window for performing drug screens to identify anti-(lymph)angiogenic compounds for developing as anti-cancer therapies.

Zebrafish cancer xenotransplantation models

With several discrepancies between developmental and tumor-associated angiogenesis with respect to morphological and molecular features [26], an ideal anti-

[1] Please inert Table 1 after this.

angiogenic drug should target only tumor-associated angiogenesis without affecting normal developmental angiogenesis. Excluding developmental angiogenesis, the zebrafish now represents an established model for investigating tumor-associated angiogenesis, first reported by Haldi and colleagues who effectively transplanted metastatic human melanoma WM-266-4 cells into 48 hpf zebrafish embryos [51]. In this study, transplanted cells were shown to rapidly proliferate, migrate, form tumor like masses and stimulate angiogenesis through recruitment of host endothelial cells and the formation of new vessels infiltrating the tumor mass by 7 days post-injection. Perhaps pertinent, the yolk sac of 2 dpf embryos was chosen to be an ideal xenotransplantation site due to its avascular and nutrient-rich environment and tumor cells injected into this site were able to stimulate cancer-associated angiogenesis primarily from subintestinal vessels. To date, cell lines from several human cancers such as melanoma [51,165], lung [106], neuroendocrine [148], colon [63,166], glioma [98,161], breast [5,55], pancreatic [50,149], prostate [24,88] and ovarian cancers [110], have all been successfully transplanted into zebrafish embryos and shown to stimulate tumor-associated angiogenesis. Importantly, human and murine cancer cell lines engineered to express pro-angiogenic factors relevant to cancer-associated angiogenesis, for example human FGF2 and/ or VEGF can also be injected to demonstrate potent angiogenic responses in zebrafish larvae, showing conservation and similarities in molecular mechanisms between zebrafish and human [110]. Notwithstanding, blood vessels are now known to play a pivotal role for tumor dissemination in zebrafish and this was elegantly demonstrated when highly metastatic human PaTu8988-T pancreatic cancer cell line transplanted into a 2 dpf *cloche* mutant embryo that lack functional vasculature, essentially failed to disseminate [103,134]. Primary human tumor models derived from prostate [8], pancreatic, gastrointestinal cancer patients [103] and primary leukaemia's [12,118] have also been reported to be transplantable into zebrafish embryos but whether they induce tumor-associated angiogenesis has not been fully investigated. Transplanting human cancers into juvenile (30 dpf) zebrafish has also been demonstrated to induce tumor associated-angiogenesis [136]. Although a key limitation in juvenile xenotransplantation model is the need for artificial suppression of the host adaptive immunity which is already well established and fully functional [89], it is important to note that ectopic vessels formed in these juvenile xenotransplantation model is likely to be closer to its human counterpart, as they originate from fully developed blood vessels that include smooth muscle cells and

pericytes [128]. In contrast, it remains unclear if tumor-associated angiogenesis observed in embryonic zebrafish results from redirection of developing vasculature. In this regard, the recently generated double mutant line *recombinant activated gene* 2 (*rag2*)^{f450s} (*casper*) mutant that is immunocompromised and transparent at juvenile stage will be useful for future xenotransplantation studies using juvenile fish [139,140,155].

Automated platforms

Finally in this section, to facilitate rapid identification of anti-(lymph)angiogenic drugs, the transgenic lines as listed in Table 1, can be combined with mechanical devices to allow fully automated testing of large number of chemicals [97,144]. For example, an innovative design for high-throughput microfluidic chip-based device was recently developed for automated immobilization and timelapse imaging of developing zebrafish embryos under continuous microperfusion of embryonic medium [172]. The chip design facilitates rapid loading and immobilization of up to 252 living embryos in 12 clusters. Importantly, Zhu and colleague demonstrated that the chip can be used to identify anti-angiogenic drugs using the *Tg(fli1a:EGFP)*^{y1} transgenic without the need for manual quantification of intersegmental vessel development.

Discoveries of novel anti-vascular drugs using zebrafish

Due to many advantages of the zebrafish platform, research groups have embraced the utility of the model to identify novel anti-vascular drugs with anti-cancer properties and notable examples are listed in Table 2[2]. For example, Wang and colleagues used *Tg(kdrl:EGFP)*^{s843} transgenic embryos to screen ~2000 compounds from the Spectrum Collection compound library and identified seven that demonstrated inhibition of intersegmental vessel development in 52 hpf zebrafish embryos when exposed through bathing [151]. They subsequently showed that treatment with one of these drugs rosuvastatin, a synthetic statin commonly used for treatment of dyslipidemia, significantly reduced tumor-associated angiogenesis and tumor volume of mouse xenografted with human PPC-1 prostate cancer cells, essentially by inhibiting tumor cell proliferation and enhancing tumor apoptosis. Similarly, Camus and colleagues screened ~288 putative kinase

[2] Please insert Table 2 after this

inhibitors from the BioFocus SoftFocus library SFK33 using *Tg(fli1a:EGFP)*^{y1} transgenic embryos and identified seven that were observed to inhibit intersegmental vessel development when exposed through bathing [18]. Further analysis using *in vitro* kinase profiling, *in vivo* mRNA rescue experiments and three dimensional modelling analysis, noted that 2 of these compounds were unexpectedly targeting the PhKG1 of PhK, a holoenzyme that regulate glycogenolysis through activation of glycogen phosphorylase. Further investigation of the association of PhKG1 and cancer revealed that the expression of this molecule was highly upregulated in various human cancers, for instance breast, colon, and kidney when compared to tissue-matched controls, highlighting the relevance of PhKG1 as a potential therapeutic target for human cancers.

In addition to conducting screens, the zebrafish platform holds value to rapidly investigate anti-angiogenic potential of novel synthetic leads. For example, bath exposure of YLT192, a novel synthetic compound with excellent pharmacokinetic profile was recently shown to inhibit intersegmental vessel development in *Tg(kdrl:EGFP)*^{zn1} embryos, largely by preventing the phosphorylation of VEGFR-2 [159]. Furthermore, in the same study, when YLT192 was given once daily by oral gavage at a dose of 100 mg/kg, significant reduction in tumor volume and tumor-associated angiogenesis of mouse xenografted with either human glioblastoma U-251 or human colon carcinoma HCT116 cells, was observed by 24 days post-treatment. Similar strategies have been utilized to identify synthetic anti-angiogenic compounds with anti-cancer properties, for example SKLB1002 [165], SKLB-329 [169], SKLB646 [168] and thalidomide analogue Gu1029 [11]. Of note, the study conducted by Beedie and colleagues which showed potent anti-angiogenic activity of thalidomide analogue Gu1029 represents an excellent example of how the zebrafish model can be used to identify more clinically relevant analogues of FDA-approved drugs associated with multiple severe side effects such as thalidomide.

While utilising the developmental angiogenesis model in zebrafish to identify novel anti-angiogenic drugs is clearly efficient and cost-effective strategy, identifying drugs that inhibit neovascularisation in the zebrafish xenotransplantation model would be more relevant to cancer treatment. A study conducted by Yang and colleagues took advantage of this xenotransplantation model and demonstrated that bath exposure of Nordy, a synthetic anti-cancer compound [14,22], was able to inhibit cancer-associated angiogenesis and tumor dissemination in human U87 glioma stem cell xenografted *Tg(fli1a:EGFP)*^{y1} zebrafish

larvae at a concentration of 50 µM, broadly by suppressing VEGF secretion from the transplanted cells and promoting differentiation into less-invasive differentiated U87 cells [161]. Importantly, developmental angiogenesis was not affected upon Nordy treatment demonstrating its exquisite specificity towards glioma stem cell-associated angiogenesis.

Compared to anti-angiogenic drugs, the zebrafish has only been recently utilized to identify novel anti-lymphangiogenic hits. In a recent study conducted by Astin and colleagues, *in situ* hybridisation of the *lyve1b* gene and lymphangiogenic analysis in *Tg(lyve1b:EGFP)*^{nz150} transgenic larvae was used to assess if any of the 1120 compounds in the Prestwick Chemical Library containing FDA-approved compounds were able to inhibit thoracic duct development in zebrafish when exposed through bathing. From this screen, the authors identified 4 novel anti-lymphangiogenic compounds [6]. Of these, a natural flavonoid, kaempferol was able to reduce lymph node metastasis in mice xenografted with MDA-MB-231 human breast cancer cells. However, kaempferol treatment was also found to increase systemic metastasis in this model, highlighting the need for combinational modality with this hit. Regardless, this study by Astin and colleagues clearly demonstrates that the zebrafish model can be used effectively to screen for anti-lymphangiogenic compounds with anti-cancer properties.

Limitations

Although the zebrafish model provides an attractive platform for identifying anti-(lymph)angiogenic drugs that may hold value as potential cancer therapeutics, there are several limitations that need to be taken into consideration. Firstly, the lack of mammalian organs such as lungs, mammary gland, and the prostate for example, limits the possibility of conducting orthotopic transplantation experiments [147]. Hence, modelling how human cancers that are native to these organs stimulate cancer-associated angiogenesis in their primary tumor site microenvironment, remains challenging. This is further exacerbated by the requirement of lower temperatures (35 ^{o}C) to maintain zebrafish transplanted with human cancer cells that require an optimal temperature of 37 ^{o}C and not forgetting that the normal temperature for zebrafish maintenance is 28 ^{o}C [51]. This can be crucial as human cancer cells may behave differently depending on their incubation temperature and therefore potential drugs identified under these conditions may not be clinically relevant [83].

Secondly, the primary method for drug exposure for drug screens using zebrafish embryos/larvae involves drug bathing, which does not permit samples with low water solubility to be effectively tested. To this end, zebrafish embryos/larvae are well tolerant to various organic solvents such as the most commonly used DMSO (up to a concentration of 1%), allowing samples that are soluble in these organic solvents to be tested [52]. Another limitation of drug bathing is the presence of the chorion in early stage embryos (pre-3 dpf), which may act as a protective barrier against high molecular weight drugs [9,74,75,79]. This can be mitigated by either dechorionating the embryos before drug treatment or via direct injection of the drug of interest into the circulatory system, however, these methods can significantly delay the screening process. Interestingly, it had been shown that DMSO at a concentration of ≥ 0.1% decreases the barrier function of the zebrafish chorion while DMSO at a concentration of 0.01% had no effects [75]. This shows value of DMSO co-treatment when using zebrafish embryos with chorion for drug screens. Also noteworthy, not all chemicals delivered through bathing can be taken up by the zebrafish embryo due to the ATP-binding cassette (ABC) transporter activity of Abcb, member 4 (Abcb4), a functional zebrafish orthologue of mammalian ABCB, member 1 (ABCB1) protein, which acts as a cellular toxicant transporter that confers resistance of embryos to ABCB1 substrates [38].

Third, development of subintestinal vessels do not require several key pro-angiogenic factors as mentioned above [46]. Hence, these molecules may not stimulate tumor-associated angiogenesis in subintestinal vessels, potentially giving rise to false negative hits when conducting anti-angiogenic screens using this site. Further studies are needed to investigate whether human cancer cells overexpressing these angiogenic factors are able to stimulate tumor-associated angiogenesis in subintestinal vessels.

Fourth, whether the molecular mechanisms involved in lymphatic specification are conserved in zebrafish still remains controversial and consequently, drugs modulating this process may not be easily identified using zebrafish. Orthologues of lymphatic genes involved in mammalian lymphatic specification, for instance *prox1a* [163], *prox1b* [32], nuclear receptor subfamily 2, group F, member 2 (*nr2f2*) [4] and SRY (sex determining region Y)-box 18 (*sox18*) [20] have all been shown to be required for zebrafish thoracic duct development using morpholino-mediated knockdown followed by lymphatic characterization of morphant larvae. However, several studies using zebrafish mutant larvae essentially demonstrated that these aforementioned genes are likely not required for

thoracic duct development [141,146]. The studies that use zebrafish mutants are more conclusive as morpholino-mediated knockdown are more sensitive to off target side effects [82]. Although a recent study counter argues this by showing that lymphatic endothelial cell number within the thoracic duct is reduced in *prox1a* mutant larvae and that maternally deposited Prox1a was likely the cause of the mild phenotype observed in the *prox1a*^{i278} mutant larvae used in the study conducted by Van Impel and colleagues [85], further studies are nevertheless required to determine the true role of these lymphatic genes in the zebrafish. It is important to note though that mechanisms involved in lymphatic sprouting and migration have been shown to be highly conserved in zebrafish [84,107] and thus, this still remains a reliable platform for identifying anti-lymphangiogenic drugs that affect lymphatic sprouting and migration.

Finally, lymph node lymphangiogenesis cannot be modelled in zebrafish, essentially as lymph nodes have yet to be identified. In contrast, in human there are ~600 lymph nodes and are all fully characterized [164] and pertinently, in a disease state, tumor cells are now known to stimulate lymph node lymphangiogenesis even before metastasizing and this can result in increased tumor metastasis to distant sites [56,59,60]. Hence, the zebrafish model is severely limited to identify drugs that might target this important process. The development of various transgenic zebrafish that label lymphatics (see Table 1), antigen presenting cells [36,48,126,150,157], and T- and B-cells [90,91,115] should encourage future studies on investigating whether a functional lymph node likely develops and is present, in juvenile and adult zebrafish, respectively.

Future directions

Despite various limitations as mentioned above, the zebrafish model possess unique features that make it an ideal complementary model to experimental mouse models. For example, the zebrafish model can enable the simultaneous study of developmental and tumor-associated angiogenesis. The ability to distinguish drugs that can target tumor-angiogenesis but not developmental angiogenesis in a relatively short time is a very powerful and unique advantage of using the zebrafish model [11]. Not only does this allow identification of drugs that potentially have minimal side effects but may also result in the identification of those hits that target pathways outside of the VEGFR-2 pathway, essential component for developmental angiogenesis in zebrafish. This is particularly important as

majority of clinically approved anti-angiogenic drugs focus on VEGFR-2 pathway inhibition and cancer patients treated with these drugs, as mentioned above, have been reported to develop resistance [13,43]. Therefore, future work could focus on identifying drugs that target compensatory angiogenic pathways and mechanisms to improve effectiveness of currently available anti-angiogenic therapies.

Although tumor-associated angiogenesis models are well established in zebrafish, whether cancer xenotransplantation in zebrafish also induces tumor-associated lymphangiogenesis is currently unknown. To this end, we have shown in a recent study that by inducing inflammation using 2,4,6-trinitrobenzenesulfonic acid (TNBS), stimulated macrophage-dependent inflammatory lymphangiogenesis in the intestinal lymphatic vessels in embryonic zebrafish [113], demonstrating the utility of the zebrafish for investigating pathological lymphangiogenesis. As discussed above, a lack of a well characterized lymph nodes and the controversies surrounding the molecular basis involved in lymphatic specification in zebrafish, currently limits our ability to model tumor-associated lymphangiogenesis in this vertebrate system. Regardless, a zebrafish model of tumor-associated lymphangiogenesis can hold great value in identifying potential therapeutic hits that can specifically target tumor-associated lymphatic sprouting and migration and in elucidating differences in molecular mechanisms between tumor-associated and developmental lymphangiogenesis. Hence, future work focusing on establishing tumor-associated lymphangiogenesis models in larval/juvenile/adult zebrafish using established xenotransplantation protocols and lymphatic zebrafish transgenic lines should be encouraged.

Tumor intravasation and extravasation into and from tumor-associated vasculature represents an important event in tumor metastasis [122]. However, compared to tumor-associated (lymph)angiogenesis, the molecular mechanisms involved in these important processes are not well elucidated. This is partly due to the lack of models that allow robust *in vivo* analysis enabling real time monitoring of these events. Due to the optical transparency of zebrafish embryos and the availability of vascular transgenic lines, the zebrafish xenotransplantation platform has been successfully used to study all different stages of metastatic processes in blood vessels [23,103,135,140,162]. This unique opportunity should be utilized to screen for drugs that can inhibit these important processes and to further understand their molecular mechanisms. For example, a recent study by

Chen and colleagues showed that 1) orthotopic xenotransplantation of mouse SJmRBL-8 retinoblastoma cells into 2 dpf zebrafish embryos results in the formation of tumor clusters around the blood vessels attached to the lens (hyaloid vessels), and 2) Sunitinib treatment and morpholino-mediated *vegfaa* knockdown attenuates retinoblastoma invasion and metastasis in this model [23]. This suggests anti-angiogenic treatment as an attractive therapeutic option for retinoblastoma in human patients.

Conclusion

New and emerging tools and technologies such as the CRISPR/Cas9 system [68], various cancer xenotransplantation models [147], and the development of many vascular-specific transgenics [85,112] are continuously expanding the capabilities of the zebrafish model as a drug discovery platform. As of now, majority of anti-(lymph)angiogenic screens in zebrafish focus on developmental (lymph)angiogenesis but future work should incorporate all of the above to identify drugs that are more relevant to human tumor-associated (lymph)angiogenesis. Pre-clinical testing using widely used mammalian cancer models will continue to be a gold standard in the drug discovery field. However, with the recent emergence of human cancer patient-derived tissue/cell xenotransplantation in zebrafish [8,12,103,118,152], and the generation of transparent and immunocompromised mutant zebrafish line that readily enable cancer cell xenotransplantation into juvenile fish [140], we foresee the zebrafish model playing a bigger role as an excellent complementary model in not only drug discovery but in pre-clinical drug evaluations as well.

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Ethics approval

All experiments were done in accordance to UKM-IACUC.

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Conflict of interest

The authors declare that they have no competing interests.

Table 1: Promoter genes used in generating transgenic lines for vascular research

tgGenes	Transgenic line examples	Vascular endothelial expression	Other expression sites	References
cdh5	*TgBAC(cdh5:GAL4FF)*^{mu101}	Blood vessels		[17]
etv2	*Tg(etsrp:EGFP)*^{ci1}	Blood vessels	Neural tube	[117]
fli1a	*Tg(fli1a: EGFP)*^{y1} *Tg(fli1a: nEGFP)*^{sd2}	Blood and lymphatic vessels	Myeloid cells, neural crest cells and muscle	[93,123]
flt1	*Tg(-0.8flt1:RFP)*^{hu5333} *Tg(flt1: YFP)*^{hu4624}	Blood vessels with strong arterial expression		[16,62]
flt4	*TgBAC(flt4; Citrine)*^{hu7135} *Tg(flt4:YFP)*^{hu4881}	Initially marks all blood vessels but expression becomes progressively restricted to the veins from 26 hpf. Lymphatic vessels		[62,146]
kdrl (flk1)	*Tg(kdrl:EGFP)*^{s843} *Tg(kdrl: RFP)*^{la4}	Blood vessels	Endodermal pouches	[66,73]
lyve1b	*Tg(-5.2lyve1b:DsRed)*^{nz101} *Tg(-5.2lyve1b:EGFP)*^{nz150}	Lymphatic vessels and primary veins		[112]
prox1a	*TgBAC(prox1a:KalTA4-4xUAS-E1b:unctagRFP)*^{nim5} *TgBAC(prox1a:Citrine)*^{zf338}	Lymphatic vessels and lymphatic precursor cells on the posterior cardinal vein	Lens, retina, liver, neuromasts and myotome	[34,85,146]

SAGFF27C	*SAGFF27C;UAS:GFP*	Lymphatic vessels	Hematopoietic cells, neurons	[16]
stab1	*TgBAC(stab1:YFP)^{hu4453}*	Lymphatic vessels and primary veins		[61]

BAC: Bacterial artificial chromosome, cdh5: Cadherin 5, DsRed: Discosoma sp. Red fluorescent protein, EGFP: Enhanced green fluorescent protein, etv2: ets variant, fli1a: Fli-1 proto-oncogene, ETS transcription factor a, flt1: Fms-related tyrosine kinase 1/vascular endothelial growth factor 1, flt4: Fms-related tyrosine kinase 4/ vascular endothelial growth factor 3, kdrl : Kinase insert domain receptor like/vascular endothelial growth factor 2, lyve1b: Lymphatic vessel endothelial hyaluronic receptor 1b, nEGFP: Nuclear EGFP, prox1a: Prospero homeobox 1a, RFP: Red fluorescent protein, stab1: Stabilin 1, YFP: Yellow fluorescent protein.

Table 2: Novel anti-(lymph)angiogenic drugs with *in vivo* anti-cancer property identified using zebrafish

Compound	Molecular target	Cancer	Phenotype	Reference
Barbigerone isolated from *Tephrosia barbigera*	AKT, FAK, MAPK	Murine B16-F10 melanoma cell line	Inhibits ISV and dorsal anastomotic vessel development, melanoma-associated angiogenesis and disturbs vessel annulation in fins	[160]
Closantel	VEGFR	Human HepG2 hepatocellular carcinoma cell line, human PANC-1 pancreatic carcinoma cell line, human HeLa cervical carcinoma cell line, human Ramos Burkitt's lymphoma cell line	Inhibits ISV and SIV development	[173]
Deoxypodophyllotoxin from from *Anthriscus*	AMPK	Human SGC-7901 gastric	Inhibits ISV development	[153]

sylvestris		cancer cell line		
DHFR-F56 chimeric protein	PI3K-AKT	Human HT-29 colorectal adenocarcinoma and BGC-823 gastric carcinoma cell lines	Inhibits SIV development	[171]
DMH1	BMP	A549 human lung adenocarcinoma cell line and MMTV-PyVmT transgenic mice for human metastatic breast cancer	Inhibits SIV development	[46,53,54,64,114]
Furanodiene isolated from *Curcuma wenyujin*	AKT, PIK3R1, ERK	Human MDA-MB-231 breast cancer and HeLa cervical cancer cell lines	Inhibits ISV development	[101,138,170]
Gu1029	Not investigated	Human PC3 prostate cancer cell line	Inhibits ISV development	[11]
Kaempferol	VEGFR-2/3	Human MDA-MB-231 breast cancer cell line	Inhibits PL and TD development	[6]
Nordy	$VEGF^{165}$ secretion	Glioma stem cells from human U87 cell	Inhibits glioma stem cell-associated	[161]

		line	angiogenesis	
Noreremophilane 11	VEGFR-2	Human MDA-MB-231 breast cancer	Inhibits SIV development and breast cancer associated-angiogenesis	[108]
Quercetin (micelle-encapsulated quercetin)	VEGFR-2, ERK	Various cancers including melanoma, oesophageal, ovarian, liver and breast cancers	Inhibits ISV, DA and PCV development	[158,167] (reviewed in [45])
Raddeanin A isolated from *Anemone raddeana*	VEGFR-2	Human HCT-15 colon adenocarcinoma	Inhibits ISV development	[49]
Recombinant protein HB-002.1	VEGF-A	Human Colo-205 colon adenocarcinoma and A549 human lung adenocarcinoma cell lines	Inhibits SIV development	[102]
Rosuvastatin	Not investigated	Human PPC-1 prostate cancer cell line	Inhibits ISV development	[151]
SKLB1002	VEGFR-2	Murine B16-F10 melanoma, 4T1 mammary cancer and CT26	Inhibits ISV development and melanoma-	[165]

		colon carcinoma cell lines	associated angiogenesis	
SKLB329	VEGFR1/2/3, FGFR2, SRC	Human Hep G2 and SMMC7721 hepatocellular carcinoma cell lines	Inhibits ISV development	[169]
SKLB646	SRC/RAF/VEGFR2	Human MDA-MB-231, MDA-MB-435 triple negative breast cancer cell lines	Inhibits ISV development	[168]
3,4',5-trans-Trimethoxestilbene	VEGFR-2	Various cancers including prostate, lung, colon, liver and breast cancers	Inhibits ISV development	[3] (reviewed in [1])
YLT1962	VEGFR-2	Human U-251 glioblastoma and HCT-116 colon cancer cell lines	Inhibits ISV development	[159]

AKT: Protein kinase B, AMPK: 5' adenosine monophosphate-activated protein kinase, BMP: Bone morphogenetic protein, DA: Dorsal aorta, ERK: extracellular signal-regulated kinase, FAK: Focal adhesion kinase, FGFR: Fibroblast growth factor receptor, ISV: intersegmental vessel, MAPK: Mitogen-activated protein kinase, PCV: Posterior cardinal vein, PIK3R1: Phosphatidylinositol 3-kinase regulatory subunit alpha, PI3K: Phosphoinositide 3-kinase, PL: Parachordal lymphangioblast, RAF: RAF proto-oncogene serine/threonine-protein kinase, SIV: Subintestinal vessel, SRC: SRC proto-oncogene, non-receptor tyrosine kinase, TD: thoracic duct, VEGFR: Vascular endothelial growth factor receptor,

Figure 1: Vascular development and quantification in zebrafish

(A) Lateral image of the *Tg(fli1a:EGFP)*^{y1} transgenic at 72 hpf showing the locations of ISV, SIV and PL. (B) Lateral image of the *Tg(lyve1b:dsRed2)*^{nz101} transgenic at 6 dpf showing the location of the TD. (C-F') Lateral images of the ISV (purple) in the *Tg(fli1a:EGFP)*^{y1} transgenic at 30 hpf (C,D) and 48 hpf (E,F) treated with either 0.5% DMSO (C,E) or 20 µM sunitinib malate (D,F) and their respective schematic images (C'-F'). Only ISV located on the right side of the fish are coloured 'in red'. ISV development can be quantified by either determining the proportion of fully developed and mature ISVs (black asterisks) or by calculating the average lengths of the ISVs (measured as depicted by black dotted arrows). Treatment with 20 µM sunitinib malate at 16 hpf results in impaired ISV development at both 30 and 48 hpf (D-D',F-F'). (G-H') Lateral images of the SIV (purple) in the *Tg(fli1a:EGFP)*^{y1} transgenic at 72 hpf treated with either 0.5% DMSO (G) or 20 µM sunitinib malate (H) and their respective schematic images (G',H'). SIV development can be quantified by either counting the number of interconnecting vessels (marked with black asterisks),

quantifying the size of the SIVs (measured as shown by green dotted lines) or by counting the number of vascular compartments within the SIVs (marked with numbers). Treatment with 20 µM sunitinib malate at 16 hpf results in impaired SIV development at 72 hpf (H,H'). (I-J') Lateral images of the PL (purple) in the *Tg(fli1a:EGFP)*^{y1} transgenic at 54 hpf treated with either 0.5% DMSO (I) or 15 µM sunitinib malate (J) and their respective schematic images (I',J'). PL development can be quantified by counting the number of somites (black dotted arrows) with PLs (as marked with black asterisks). Treatment with 15 µM sunitinib malate at 24 hpf results in a lack of PL development at 54 hpf (J,J'). (K-L') Lateral images of the TD (purple) in the *Tg(lyve1b:DsRed2)*^{nz101} transgenic at 6 dpf treated with either 0.5% DMSO (K) or 15 µM sunitinib malate (L) and their respective schematic images (K',L'). TD development can be quantified by either counting the number of somites (black dotted arrows) with complete TD (as marked with black asterisks) or by counting the number of lymphatic endothelial cells within the zebrafish TD (marked with numbers). Treatment with 15 µM sunitinib malate at 24 hpf results in a lack of TD development at 6 dpf (L,L').Fluorescent images were taken using either the Olympus MVX10 fluorescent microscope or z stacks 5 µm apart using the Olympus IX81 fluorescent microscope and were processed using ImageJ [121]. DA, dorsal aorta; DLAV, dorsal longitudinal anastomotic vessel; DLLV, dorsal longitudinal lymphatic vessel; DMSO, dimethyl sulfoxide, dpf, days post-fertilisation; ISLV, intersegmental lymphatic vessel; ISV, intersegmental vessel; PCV, posterior cardinal vein; PL, parachordal lymphangioblast; SIV, subintestinal vessel; TD, thoracic duct. Scale bars: 100 µm.

Reference

1. Aldawsari FS, Velazquez-Martinez CA. 3,4',5-trans-Trimethoxystilbene; a natural analogue of resveratrol with enhanced anticancer potency. *Investigational new drugs* **33**: 775-786, 2015.

2. Alders M, Hogan BM, Gjini E, Salehi F, Al-Gazali L, Hennekam EA, Holmberg EE, Mannens MM, Mulder MF, Offerhaus GJ, Prescott TE, Schroor EJ, Verheij JB, Witte M, Zwijnenburg PJ, Vikkula M, Schulte-Merker S, Hennekam RC. Mutations in CCBE1 cause generalized lymph vessel dysplasia in humans. *Nature genetics* **41**: 1272-1274, 2009.

3. Alex D, Leong EC, Zhang ZJ, Yan GT, Cheng SH, Leong CW, Li ZH, Lam KH, Chan SW, Lee SM. Resveratrol derivative, trans-3,5,4'-trimethoxystilbene, exerts antiangiogenic and vascular-disrupting effects in zebrafish through the downregulation of VEGFR2 and cell-cycle modulation. *Journal of cellular biochemistry* **109**: 339-346, 2010.

4. Aranguren XL, Beerens M, Vandevelde W, Dewerchin M, Carmeliet P, Luttun A. Transcription factor COUP-TFII is indispensable for venous and lymphatic development in zebrafish and Xenopus laevis. *Biochemical and biophysical research communications* **410**: 121-126, 2011.

5. Astin JW, Haggerty MJ, Okuda KS, Le Guen L, Misa JP, Tromp A, Hogan BM, Crosier KE, Crosier PS. Vegfd can compensate for loss of Vegfc in zebrafish facial lymphatic sprouting. *Development* **141**: 2680-2690, 2014.

6. Astin JW, Jamieson SM, Eng TC, Flores MV, Misa JP, Chien A, Crosier KE, Crosier PS. An in vivo antilymphatic screen in zebrafish identifies novel inhibitors of mammalian

lymphangiogenesis and lymphatic-mediated metastasis. *Molecular cancer therapeutics* **13**: 2450-2462, 2014.

7. Baldwin ME, Tester AM, Phelan D, Klupacs R. The novel therapeutic monoclonal antibody VGX-100 neutralises VEGF-C and inhibits tumor growth and metastasis in subcutaneous and orthotopic models of human cancer. *AACR; Cancer Research* **71(8 Supplement)**: LB-24, 2011.

8. Bansal N, Davis S, Tereshchenko I, Budak-Alpdogan T, Zhong H, Stein MN, Kim IY, Dipaola RS, Bertino JR, Sabaawy HE. Enrichment of human prostate cancer cells with tumor initiating properties in mouse and zebrafish xenografts by differential adhesion. *The Prostate* **74**: 187-200, 2014.

9. Bar-Ilan O, Louis KM, Yang SP, Pedersen JA, Hamers RJ, Peterson RE, Heideman W. Titanium dioxide nanoparticles produce phototoxicity in the developing zebrafish. *Nanotoxicology* **6**: 670-679, 2012.

10. Beasley NJ, Prevo R, Banerji S, Leek RD, Moore J, van Trappen P, Cox G, Harris AL, Jackson DG. Intratumoral lymphangiogenesis and lymph node metastasis in head and neck cancer. *Cancer research* **62**: 1315-1320, 2002.

11. Beedie SL, Peer CJ, Pisle S, Gardner ER, Mahony C, Barnett S, Ambrozak A, Gutschow M, Chau CH, Vargesson N, Figg WD. Anticancer Properties of a Novel Class of Tetrafluorinated Thalidomide Analogues. *Molecular cancer therapeutics* **14**: 2228-2237, 2015.

12. Bentley VL, Veinotte CJ, Corkery DP, Pinder JB, LeBlanc MA, Bedard K, Weng AP, Berman JN, Dellaire G. Focused chemical genomics using zebrafish xenotransplantation as a pre-clinical therapeutic platform for T-cell acute lymphoblastic leukemia. *Haematologica* **100**: 70-76, 2015.

13. Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. *Nature reviews. Cancer* **8**: 592-603, 2008.

14. Bian XW, Xu JP, Ping YF, Wang Y, Chen JH, Xu CP, Wu YZ, Wu J, Zhou XD, Chen YS, Shi JQ, Wang JM. Unique proteomic features induced by a potential antiglioma agent, Nordy (dl-nordihydroguaiaretic acid), in glioma cells. *Proteomics* **8**: 484-494, 2008.

15. Bos FL, Caunt M, Peterson-Maduro J, Planas-Paz L, Kowalski J, Karpanen T, van Impel A, Tong R, Ernst JA, Korving J, van Es JH, Lammert E, Duckers HJ, Schulte-Merker S. CCBE1 is essential for mammalian lymphatic vascular development and enhances the lymphangiogenic effect of vascular endothelial growth factor-C in vivo. *Circulation research* **109**: 486-491, 2011.

16. Bussmann J, Bos FL, Urasaki A, Kawakami K, Duckers HJ, Schulte-Merker S. Arteries provide essential guidance cues for lymphatic endothelial cells in the zebrafish trunk. *Development* **137**: 2653-2657, 2010.

17. Bussmann J, Wolfe SA, Siekmann AF. Arterial-venous network formation during brain vascularization involves hemodynamic regulation of chemokine signaling. *Development* **138**: 1717-1726, 2011.

18. Camus S, Quevedo C, Menendez S, Paramonov I, Stouten PF, Janssen RA, Rueb S, He S, Snaar-Jagalska BE, Laricchia-Robbio L, Izpisua Belmonte JC. Identification of phosphorylase kinase as a novel therapeutic target through high-throughput screening for anti-angiogenesis compounds in zebrafish. *Oncogene* **31**: 4333-4342, 2012.

19. Cannon JE, Upton PD, Smith JC, Morrell NW. Intersegmental vessel formation in zebrafish: requirement for VEGF but not BMP signalling revealed by selective and non-selective BMP antagonists. *British journal of pharmacology* **161**: 140-149, 2010.

20. Cermenati S, Moleri S, Neyt C, Bresciani E, Carra S, Grassini DR, Omini A, Goi M, Cotelli F, Francois M, Hogan BM, Beltrame M. Sox18 genetically interacts with VegfC to regulate lymphangiogenesis in zebrafish. *Arteriosclerosis, thrombosis, and vascular biology* **33**: 1238-1247, 2013.

21. Cha YR, Fujita M, Butler M, Isogai S, Kochhan E, Siekmann AF, Weinstein BM. Chemokine signaling directs trunk lymphatic network formation along the preexisting blood vasculature. *Developmental cell* **22**: 824-836, 2012.

22. Chen JH, Bian XW, Yao XH, Gong W, Hu J, Chen K, Iribarren P, Zhao W, Zhou XD. Nordy, a synthetic lipoxygenase inhibitor, inhibits the expression of formylpeptide receptor and induces differentiation of malignant glioma cells. *Biochemical and biophysical research communications* **342**: 1368-1374, 2006.

23. Chen X, Wang J, Cao Z, Hosaka K, Jensen L, Yang H, Sun Y, Zhuang R, Liu Y, Cao Y. Invasiveness and metastasis of retinoblastoma in an orthotopic zebrafish tumor model. *Scientific reports* **5**: 10351, 2015.

24. Chiavacci E, Rizzo M, Pitto L, Patella F, Evangelista M, Mariani L, Rainaldi G. The zebrafish/tumor xenograft angiogenesis assay as a tool for screening anti-angiogenic miRNAs. *Cytotechnology* **67**: 969-975, 2015.

25. Childs S, Chen JN, Garrity DM, Fishman MC. Patterning of angiogenesis in the zebrafish embryo. *Development* **129**: 973-982, 2002.

26. Chung AS, Lee J, Ferrara N. Targeting the tumour vasculature: insights from physiological angiogenesis. *Nature reviews. Cancer* **10**: 505-514, 2010.

27. Covassin LD, Siekmann AF, Kacergis MC, Laver E, Moore JC, Villefranc JA, Weinstein BM, Lawson ND. A genetic screen for vascular mutants in zebrafish reveals dynamic roles for Vegf/Plcg1 signaling during artery development. *Developmental biology* **329**: 212-226, 2009.

28. Covassin LD, Villefranc JA, Kacergis MC, Weinstein BM, Lawson ND. Distinct genetic interactions between multiple Vegf receptors are required for development of different blood vessel types in zebrafish. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 6554-6559, 2006.

29. Coxam B, Sabine A, Bower NI, Smith KA, Pichol-Thievend C, Skoczylas R, Astin JW, Frampton E, Jaquet M, Crosier PS, Parton RG, Harvey NL, Petrova TV, Schulte-Merker S, Francois M, Hogan BM. Pkd1 regulates lymphatic vascular morphogenesis during development. *Cell reports* **7**: 623-633, 2014.

30. Cutler C, Multani P, Robbins D, Kim HT, Le T, Hoggatt J, Pelus LM, Desponts C, Chen YB, Rezner B, Armand P, Koreth J, Glotzbecker B, Ho VT, Alyea E, Isom M, Kao G, Armant M, Silberstein L, Hu P, Soiffer RJ, Scadden DT, Ritz J, Goessling W, North TE, Mendlein J, Ballen K, Zon LI, Antin JH, Shoemaker DD. Prostaglandin-modulated umbilical cord blood hematopoietic stem cell transplantation. *Blood* **122**: 3074-3081, 2013.

31. Dadras SS, Lange-Asschenfeldt B, Velasco P, Nguyen L, Vora A, Muzikansky A, Jahnke K, Hauschild A, Hirakawa S, Mihm MC, Detmar M. Tumor lymphangiogenesis predicts melanoma metastasis to sentinel lymph nodes. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* **18**: 1232-1242, 2005.

32. Del Giacco L, Pistocchi A, Ghilardi A. prox1b Activity is essential in zebrafish lymphangiogenesis. *PloS one* **5**: e13170, 2010.

33. Dieterich LC, Detmar M. Tumor lymphangiogenesis and new drug development. *Advanced drug delivery reviews* **99**: 148-160, 2016.

34. Dunworth WP, Cardona-Costa J, Bozkulak EC, Kim JD, Meadows S, Fischer JC, Wang Y, Cleaver O, Qyang Y, Ober EA, Jin SW. Bone morphogenetic protein 2 signaling negatively modulates lymphatic development in vertebrate embryos. *Circulation research* **114**: 56-66, 2014.

35. Elice F, Rodeghiero F. Side effects of anti-angiogenic drugs. *Thrombosis research* **129 Suppl 1**: S50-53, 2012.

36. Ellett F, Pase L, Hayman JW, Andrianopoulos A, Lieschke GJ. mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish. *Blood* **117**: e49-56, 2011.

37. Falchook GS, Goldman JW, Desai J, Leitch IM, Hong DS, Subbiah V, Rosen LS. A first-in-human phase I study of VGX-100, a selective anti-VEGF-C antibody, alone and in combination with bevacizumab in patients with advanced solid tumors. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **32:5s**: suppl; abstr 2524, 2014.

38. Fischer S, Kluver N, Burkhardt-Medicke K, Pietsch M, Schmidt AM, Wellner P, Schirmer K, Luckenbach T. Abcb4 acts as multixenobiotic transporter and active barrier against chemical uptake in zebrafish (Danio rerio) embryos. *BMC biology* **11**: 69, 2013.

39. Flores MV, Hall CJ, Crosier KE, Crosier PS. Visualization of embryonic lymphangiogenesis advances the use of the zebrafish model for research in cancer and lymphatic pathologies. *Developmental dynamics : an official publication of the American Association of Anatomists* **239**: 2128-2135, 2010.

40. Folkman J. Tumor angiogenesis: therapeutic implications. *The New England journal of medicine* **285**: 1182-1186, 1971.

41. Folkman J. Role of angiogenesis in tumor growth and metastasis. *Seminars in oncology* **29**: 15-18, 2002.

42. Fouquet B, Weinstein BM, Serluca FC, Fishman MC. Vessel patterning in the embryo of the zebrafish: guidance by notochord. *Developmental biology* **183**: 37-48, 1997.

43. Gacche RN. Compensatory angiogenesis and tumor refractoriness. *Oncogenesis* **4**: e153, 2015.

44. Geudens I, Herpers R, Hermans K, Segura I, Ruiz de Almodovar C, Bussmann J, De Smet F, Vandevelde W, Hogan BM, Siekmann A, Claes F, Moore JC, Pistocchi AS, Loges S, Mazzone M, Mariggi G, Bruyere F, Cotelli F, Kerjaschki D, Noel A, Foidart JM, Gerhardt H, Ny A, Langenberg T, Lawson ND, Duckers HJ, Schulte-Merker S, Carmeliet P, Dewerchin M. Role of delta-like-4/Notch in the formation and wiring of the lymphatic network in zebrafish. *Arteriosclerosis, thrombosis, and vascular biology* **30**: 1695-1702, 2010.

45. Gibellini L, Pinti M, Nasi M, Montagna JP, De Biasi S, Roat E, Bertoncelli L, Cooper EL, Cossarizza A. Quercetin and cancer chemoprevention. *Evidence-based complementary and alternative medicine : eCAM* **2011**: 591356, 2011.

46. Goi M, Childs SJ. Patterning mechanisms of the sub-intestinal venous plexus in zebrafish. *Developmental biology* **409**: 114-128, 2016.

47. Gomes FG, Nedel F, Alves AM, Nor JE, Tarquinio SB. Tumor angiogenesis and lymphangiogenesis: tumor/endothelial crosstalk and cellular/microenvironmental signaling mechanisms. *Life sciences* **92**: 101-107, 2013.

48. Gray C, Loynes CA, Whyte MK, Crossman DC, Renshaw SA, Chico TJ. Simultaneous intravital imaging of macrophage and neutrophil behaviour during inflammation using a novel transgenic zebrafish. *Thrombosis and haemostasis* **105**: 811-819, 2011.

49. Guan YY, Liu HJ, Luan X, Xu JR, Lu Q, Liu YR, Gao YG, Zhao M, Chen HZ, Fang C. Raddeanin A, a triterpenoid saponin isolated from Anemone raddeana, suppresses the angiogenesis and growth of human colorectal tumor by inhibiting VEGFR2 signaling. *Phytomedicine : international journal of phytotherapy and phytopharmacology* **22**: 103-110, 2015.

50. Guo M, Wei H, Hu J, Sun S, Long J, Wang X. U0126 inhibits pancreatic cancer progression via the KRAS signaling pathway in a zebrafish xenotransplantation model. *Oncology reports* **34**: 699-706, 2015.

51. Haldi M, Ton C, Seng WL, McGrath P. Human melanoma cells transplanted into zebrafish proliferate, migrate, produce melanin, form masses and stimulate angiogenesis in zebrafish. *Angiogenesis* **9**: 139-151, 2006.

52. Hallare A, Nagel K, Kohler HR, Triebskorn R. Comparative embryotoxicity and proteotoxicity of three carrier solvents to zebrafish (Danio rerio) embryos. *Ecotoxicology and environmental safety* **63**: 378-388, 2006.

53. Hao J, Ho JN, Lewis JA, Karim KA, Daniels RN, Gentry PR, Hopkins CR, Lindsley CW, Hong CC. In vivo structure-activity relationship study of dorsomorphin analogues identifies selective VEGF and BMP inhibitors. *ACS chemical biology* **5**: 245-253, 2010.

54. Hao J, Lee R, Chang A, Fan J, Labib C, Parsa C, Orlando R, Andresen B, Huang Y. DMH1, a small molecule inhibitor of BMP type i receptors, suppresses growth and invasion of lung cancer. *PloS one* **9**: e90748, 2014.

55. Harfouche R, Basu S, Soni S, Hentschel DM, Mashelkar RA, Sengupta S. Nanoparticle-mediated targeting of phosphatidylinositol-3-kinase signaling inhibits angiogenesis. *Angiogenesis* **12**: 325-338, 2009.

56. Harrell MI, Iritani BM, Ruddell A. Tumor-induced sentinel lymph node lymphangiogenesis and increased lymph flow precede melanoma metastasis. *The American journal of pathology* **170**: 774-786, 2007.

57. Hen G, Nicenboim J, Mayseless O, Asaf L, Shin M, Busolin G, Hofi R, Almog G, Tiso N, Lawson ND, Yaniv K. Venous-derived angioblasts generate organ-specific vessels during zebrafish embryonic development. *Development* **142**: 4266-4278, 2015.

58. Hermans K, Claes F, Vandevelde W, Zheng W, Geudens I, Orsenigo F, De Smet F, Gjini E, Anthonis K, Ren B, Kerjaschki D, Autiero M, Ny A, Simons M, Dewerchin M, Schulte-Merker S, Dejana E, Alitalo K, Carmeliet P. Role of synectin in lymphatic development in zebrafish and frogs. *Blood* **116**: 3356-3366, 2010.

59. Hirakawa S, Brown LF, Kodama S, Paavonen K, Alitalo K, Detmar M. VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. *Blood* **109**: 1010-1017, 2007.

60. Hirakawa S, Kodama S, Kunstfeld R, Kajiya K, Brown LF, Detmar M. VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. *The Journal of experimental medicine* **201**: 1089-1099, 2005.

61. Hogan BM, Bos FL, Bussmann J, Witte M, Chi NC, Duckers HJ, Schulte-Merker S. Ccbe1 is required for embryonic lymphangiogenesis and venous sprouting. *Nature genetics* **41**: 396-398, 2009.

62. Hogan BM, Herpers R, Witte M, Helotera H, Alitalo K, Duckers HJ, Schulte-Merker S. Vegfc/Flt4 signalling is suppressed by Dll4 in developing zebrafish intersegmental arteries. *Development* **136**: 4001-4009, 2009.

63. Hollenbach M, Stoll SJ, Jorgens K, Seufferlein T, Kroll J. Different regulation of physiological and tumor angiogenesis in zebrafish by protein kinase D1 (PKD1). *PloS one* **8**: e68033, 2013.

64. Hover LD, Young CD, Bhola NE, Wilson AJ, Khabele D, Hong CC, Moses HL, Owens P. Small molecule inhibitor of the bone morphogenetic protein pathway DMH1 reduces ovarian cancer cell growth. *Cancer letters* **368**: 79-87, 2015.

65. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, Collins JE, Humphray S, McLaren K, Matthews L, McLaren S, Sealy I, Caccamo M, Churcher C, Scott C, Barrett JC, Koch R, Rauch GJ, White S, Chow W, Kilian B, Quintais LT, Guerra-Assuncao JA, Zhou Y, Gu Y, Yen J, Vogel JH, Eyre T, Redmond S, Banerjee R, Chi J, Fu B, Langley E, Maguire SF, Laird GK, Lloyd D, Kenyon E, Donaldson S, Sehra H, Almeida-King J, Loveland J, Trevanion S, Jones M, Quail M, Willey D, Hunt A, Burton J, Sims S, McLay K, Plumb B, Davis J, Clee C, Oliver K, Clark R, Riddle C, Elliot D, Threadgold G, Harden G, Ware D, Begum S, Mortimore B, Kerry G, Heath P, Phillimore B, Tracey A, Corby N, Dunn M, Johnson C, Wood J, Clark S, Pelan S,

Griffiths G, Smith M, Glithero R, Howden P, Barker N, Lloyd C, Stevens C, Harley J, Holt K, Panagiotidis G, Lovell J, Beasley H, Henderson C, Gordon D, Auger K, Wright D, Collins J, Raisen C, Dyer L, Leung K, Robertson L, Ambridge K, Leongamornlert D, McGuire S, Gilderthorp R, Griffiths C, Manthravadi D, Nichol S, Barker G, Whitehead S, Kay M, Brown J, Murnane C, Gray E, Humphries M, Sycamore N, Barker D, Saunders D, Wallis J, Babbage A, Hammond S, Mashreghi-Mohammadi M, Barr L, Martin S, Wray P, Ellington A, Matthews N, Ellwood M, Woodmansey R, Clark G, Cooper J, Tromans A, Grafham D, Skuce C, Pandian R, Andrews R, Harrison E, Kimberley A, Garnett J, Fosker N, Hall R, Garner P, Kelly D, Bird C, Palmer S, Gehring I, Berger A, Dooley CM, Ersan-Urun Z, Eser C, Geiger H, Geisler M, Karotki L, Kirn A, Konantz J, Konantz M, Oberlander M, Rudolph-Geiger S, Teucke M, Lanz C, Raddatz G, Osoegawa K, Zhu B, Rapp A, Widaa S, Langford C, Yang F, Schuster SC, Carter NP, Harrow J, Ning Z, Herrero J, Searle SM, Enright A, Geisler R, Plasterk RH, Lee C, Westerfield M, de Jong PJ, Zon LI, Postlethwait JH, Nusslein-Volhard C, Hubbard TJ, Roest Crollius H, Rogers J, Stemple DL. The zebrafish reference genome sequence and its relationship to the human genome. *Nature* **496**: 498-503, 2013.

66. Huang H, Zhang B, Hartenstein PA, Chen JN, Lin S. NXT2 is required for embryonic heart development in zebrafish. *BMC developmental biology* **5**: 7, 2005.

67. Huth JF, Eilber FR. Patterns of metastatic spread following resection of extremity soft-tissue sarcomas and strategies for treatment. *Seminars in surgical oncology* **4**: 20-26, 1988.

68. Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR, Joung JK. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nature biotechnology* **31**: 227-229, 2013.

69. Isogai S, Horiguchi M, Weinstein BM. The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. *Developmental biology* **230**: 278-301, 2001.

70. Isogai S, Lawson ND, Torrealday S, Horiguchi M, Weinstein BM. Angiogenic network formation in the developing vertebrate trunk. *Development* **130**: 5281-5290, 2003.

71. Jeltsch M, Jha SK, Tvorogov D, Anisimov A, Leppanen VM, Holopainen T, Kivela R, Ortega S, Karpanen T, Alitalo K. CCBE1 enhances lymphangiogenesis via A disintegrin and metalloprotease with thrombospondin motifs-3-mediated vascular endothelial growth factor-C activation. *Circulation* **129**: 1962-1971, 2014.

72. Jennbacken K, Vallbo C, Wang W, Damber JE. Expression of vascular endothelial growth factor C (VEGF-C) and VEGF receptor-3 in human prostate cancer is associated with regional lymph node metastasis. *The Prostate* **65**: 110-116, 2005.

73. Jin SW, Beis D, Mitchell T, Chen JN, Stainier DY. Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. *Development* **132**: 5199-5209, 2005.

74. Johnson A, Carew E, Sloman KA. The effects of copper on the morphological and functional development of zebrafish embryos. *Aquatic toxicology* **84**: 431-438, 2007.

75. Kais B, Schneider KE, Keiter S, Henn K, Ackermann C, Braunbeck T. DMSO modifies the permeability of the zebrafish (Danio rerio) chorion-implications for the fish embryo test (FET). *Aquatic toxicology* **140-141**: 229-238, 2013.

76. Kamei M, Isogai S, Pan W, Weinstein BM. Imaging blood vessels in the zebrafish. *Methods in cell biology* **100**: 27-54, 2010.

77. Kartopawiro J, Bower NI, Karnezis T, Kazenwadel J, Betterman KL, Lesieur E, Koltowska K, Astin J, Crosier P, Vermeren S, Achen MG, Stacker SA, Smith KA, Harvey NL, Francois M,

Hogan BM. Arap3 is dysregulated in a mouse model of hypotrichosis-lymphedema-telangiectasia and regulates lymphatic vascular development. *Human molecular genetics* **23**: 1286-1297, 2014.

78. Kim JD, Kang Y, Kim J, Papangeli I, Kang H, Wu J, Park H, Nadelmann E, Rockson SG, Chun HJ, Jin SW. Essential role of Apelin signaling during lymphatic development in zebrafish. *Arteriosclerosis, thrombosis, and vascular biology* **34**: 338-345, 2014.

79. Kim KT, Tanguay RL. The role of chorion on toxicity of silver nanoparticles in the embryonic zebrafish assay. *Environmental health and toxicology* **29**: e2014021, 2014.

80. Kodera Y, Katanasaka Y, Kitamura Y, Tsuda H, Nishio K, Tamura T, Koizumi F. Sunitinib inhibits lymphatic endothelial cell functions and lymph node metastasis in a breast cancer model through inhibition of vascular endothelial growth factor receptor 3. *Breast cancer research : BCR* **13**: R66, 2011.

81. Koenig AL, Baltrunaite K, Bower NI, Rossi A, Stainier DY, Hogan BM, Sumanas S. Vegfa signaling promotes zebrafish intestinal vasculature development through endothelial cell migration from the posterior cardinal vein. *Developmental biology* **411**: 115-127, 2016.

82. Kok FO, Shin M, Ni CW, Gupta A, Grosse AS, van Impel A, Kirchmaier BC, Peterson-Maduro J, Kourkoulis G, Male I, DeSantis DF, Sheppard-Tindell S, Ebarasi L, Betsholtz C, Schulte-Merker S, Wolfe SA, Lawson ND. Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. *Developmental cell* **32**: 97-108, 2015.

83. Kokolus KM, Capitano ML, Lee CT, Eng JW, Waight JD, Hylander BL, Sexton S, Hong CC, Gordon CJ, Abrams SI, Repasky EA. Baseline tumor growth and immune control in laboratory

mice are significantly influenced by subthermoneutral housing temperature. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 20176-20181, 2013.

84. Koltowska K, Betterman KL, Harvey NL, Hogan BM. Getting out and about: the emergence and morphogenesis of the vertebrate lymphatic vasculature. *Development* **140**: 1857-1870, 2013.

85. Koltowska K, Lagendijk AK, Pichol-Thievend C, Fischer JC, Francois M, Ober EA, Yap AS, Hogan BM. Vegfc Regulates Bipotential Precursor Division and Prox1 Expression to Promote Lymphatic Identity in Zebrafish. *Cell reports* **13**: 1828-1841, 2015.

86. Kuchler AM, Gjini E, Peterson-Maduro J, Cancilla B, Wolburg H, Schulte-Merker S. Development of the zebrafish lymphatic system requires VEGFC signaling. *Current biology : CB* **16**: 1244-1248, 2006.

87. Kuo MW, Wang CH, Wu HC, Chang SJ, Chuang YJ. Soluble THSD7A is an N-glycoprotein that promotes endothelial cell migration and tube formation in angiogenesis. *PloS one* **6**: e29000, 2011.

88. Kuroyanagi J, Shimada Y, Zhang B, Ariyoshi M, Umemoto N, Nishimura Y, Tanaka T. Zinc finger MYND-type containing 8 promotes tumour angiogenesis via induction of vascular endothelial growth factor-A expression. *FEBS letters* **588**: 3409-3416, 2014.

89. Lam SH, Chua HL, Gong Z, Lam TJ, Sin YM. Development and maturation of the immune system in zebrafish, Danio rerio: a gene expression profiling, in situ hybridization and immunological study. *Developmental and comparative immunology* **28**: 9-28, 2004.

90. Langenau DM, Ferrando AA, Traver D, Kutok JL, Hezel JP, Kanki JP, Zon LI, Look AT, Trede NS. In vivo tracking of T cell development, ablation, and engraftment in transgenic zebrafish. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 7369-7374, 2004.

91. Langenau DM, Traver D, Ferrando AA, Kutok JL, Aster JC, Kanki JP, Lin S, Prochownik E, Trede NS, Zon LI, Look AT. Myc-induced T cell leukemia in transgenic zebrafish. *Science* **299**: 887-890, 2003.

92. Lawson ND, Vogel AM, Weinstein BM. sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. *Developmental cell* **3**: 127-136, 2002.

93. Lawson ND, Weinstein BM. In vivo imaging of embryonic vascular development using transgenic zebrafish. *Developmental biology* **248**: 307-318, 2002.

94. Le Guen L, Karpanen T, Schulte D, Harris NC, Koltowska K, Roukens G, Bower NI, van Impel A, Stacker SA, Achen MG, Schulte-Merker S, Hogan BM. Ccbe1 regulates Vegfc-mediated induction of Vegfr3 signaling during embryonic lymphangiogenesis. *Development* **141**: 1239-1249, 2014.

95. Lenard A, Daetwyler S, Betz C, Ellertsdottir E, Belting HG, Huisken J, Affolter M. Endothelial cell self-fusion during vascular pruning. *PLoS biology* **13**: e1002126, 2015.

96. Leslie JD, Ariza-McNaughton L, Bermange AL, McAdow R, Johnson SL, Lewis J. Endothelial signalling by the Notch ligand Delta-like 4 restricts angiogenesis. *Development* **134**: 839-844, 2007.

97. Letamendia A, Quevedo C, Ibarbia I, Virto JM, Holgado O, Diez M, Izpisua Belmonte JC, Callol-Massot C. Development and validation of an automated high-throughput system for zebrafish in vivo screenings. *PloS one* **7**: e36690, 2012.

98. Li D, Li XP, Wang HX, Shen QY, Li XP, Wen L, Qin XJ, Jia QL, Kung HF, Peng Y. VEGF induces angiogenesis in a zebrafish embryo glioma model established by transplantation of human glioma cells. *Oncology reports* **28**: 937-942, 2012.

99. Li S, Sun Y, Gao D. Role of the nervous system in cancer metastasis. *Oncology letters* **5**: 1101-1111, 2013.

100. Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. *Nature reviews. Genetics* **8**: 353-367, 2007.

101. Lim CB, Ky N, Ng HM, Hamza MS, Zhao Y. Curcuma wenyujin extract induces apoptosis and inhibits proliferation of human cervical cancer cells in vitro and in vivo. *Integrative cancer therapies* **9**: 36-49, 2010.

102. Liu L, Yu H, Huang X, Tan H, Li S, Luo Y, Zhang L, Jiang S, Jia H, Xiong Y, Zhang R, Huang Y, Chu CC, Tian W. A novel engineered VEGF blocker with an excellent pharmacokinetic profile and robust anti-tumor activity. *BMC cancer* **15**: 170, 2015.

103. Marques IJ, Weiss FU, Vlecken DH, Nitsche C, Bakkers J, Lagendijk AK, Partecke LI, Heidecke CD, Lerch MM, Bagowski CP. Metastatic behaviour of primary human tumours in a zebrafish xenotransplantation model. *BMC cancer* **9**: 128, 2009.

104. Martell RE, Chiorean EG, Youssoufian H, Rutstein MD, Pytowski B, Benjamin L, Abad L, Patel J, Steele T, Dowd M, Qin A, Kukel CF, Fox FE, Dontabhaktuni A, Morozov A. Phase I

study of the anti-VEGFR-3 monoclonal antibody IMC-3C5 in subjects with advanced solid tumors refractory to standard therapy or for which no standard therapy is available. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **29**: (suppl; abstr TPS150), 2011.

105. Mehlen P, Puisieux A. Metastasis: a question of life or death. *Nature reviews. Cancer* **6**: 449-458, 2006.

106. Moshal KS, Ferri-Lagneau KF, Haider J, Pardhanani P, Leung T. Discriminating different cancer cells using a zebrafish in vivo assay. *Cancers* **3**: 4102-4113, 2011.

107. Mulligan TS, Weinstein BM. Emerging from the PAC: studying zebrafish lymphatic development. *Microvascular research* **96**: 23-30, 2014.

108. Muthukumarasamy KM, Handore KL, Kakade DN, Shinde MV, Ranjan S, Kumar N, Sehrawat S, Sachidanandan C, Reddy DS. Identification of noreremophilane-based inhibitors of angiogenesis using zebrafish assays. *Organic & biomolecular chemistry* **14**: 1569-1578, 2016.

109. Nicenboim J, Malkinson G, Lupo T, Asaf L, Sela Y, Mayseless O, Gibbs-Bar L, Senderovich N, Hashimshony T, Shin M, Jerafi-Vider A, Avraham-Davidi I, Krupalnik V, Hofi R, Almog G, Astin JW, Golani O, Ben-Dor S, Crosier PS, Herzog W, Lawson ND, Hanna JH, Yanai I, Yaniv K. Lymphatic vessels arise from specialized angioblasts within a venous niche. *Nature* **522**: 56-61, 2015.

110. Nicoli S, Ribatti D, Cotelli F, Presta M. Mammalian tumor xenografts induce neovascularization in zebrafish embryos. *Cancer research* **67**: 2927-2931, 2007.

111. North TE, Goessling W, Walkley CR, Lengerke C, Kopani KR, Lord AM, Weber GJ, Bowman TV, Jang IH, Grosser T, Fitzgerald GA, Daley GQ, Orkin SH, Zon LI. Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. *Nature* **447**: 1007-1011, 2007.

112. Okuda KS, Astin JW, Misa JP, Flores MV, Crosier KE, Crosier PS. lyve1 expression reveals novel lymphatic vessels and new mechanisms for lymphatic vessel development in zebrafish. *Development* **139**: 2381-2391, 2012.

113. Okuda KS, Misa JP, Oehlers SH, Hall CJ, Ellett F, Alasmari S, Lieschke GJ, Crosier KE, Crosier PS, Astin JW. A zebrafish model of inflammatory lymphangiogenesis. *Biology open* **4**: 1270-1280, 2015.

114. Owens P, Pickup MW, Novitskiy SV, Giltnane JM, Gorska AE, Hopkins CR, Hong CC, Moses HL. Inhibition of BMP signaling suppresses metastasis in mammary cancer. *Oncogene* **34**: 2437-2449, 2015.

115. Page DM, Wittamer V, Bertrand JY, Lewis KL, Pratt DN, Delgado N, Schale SE, McGue C, Jacobsen BH, Doty A, Pao Y, Yang H, Chi NC, Magor BG, Traver D. An evolutionarily conserved program of B-cell development and activation in zebrafish. *Blood* **122**: e1-11, 2013.

116. Paul SM, Mytelka DS, Dunwiddie CT, Persinger CC, Munos BH, Lindborg SR, Schacht AL. How to improve R&D productivity: the pharmaceutical industry's grand challenge. *Nature reviews. Drug discovery* **9**: 203-214, 2010.

117. Proulx K, Lu A, Sumanas S. Cranial vasculature in zebrafish forms by angioblast cluster-derived angiogenesis. *Developmental biology* **348**: 34-46, 2010.

118. Pruvot B, Jacquel A, Droin N, Auberger P, Bouscary D, Tamburini J, Muller M, Fontenay M, Chluba J, Solary E. Leukemic cell xenograft in zebrafish embryo for investigating drug efficacy. *Haematologica* **96**: 612-616, 2011.

119. Ran S, Volk L, Hall K, Flister MJ. Lymphangiogenesis and lymphatic metastasis in breast cancer. *Pathophysiology : the official journal of the International Society for Pathophysiology / ISP* **17**: 229-251, 2010.

120. Ranieri G, Patruno R, Ruggieri E, Montemurro S, Valerio P, Ribatti D. Vascular endothelial growth factor (VEGF) as a target of bevacizumab in cancer: from the biology to the clinic. *Current medicinal chemistry* **13**: 1845-1857, 2006.

121. Rasband WS. ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/,. 1997-2012.

122. Reymond N, d'Agua BB, Ridley AJ. Crossing the endothelial barrier during metastasis. *Nature reviews. Cancer* **13**: 858-870, 2013.

123. Roman BL, Pham VN, Lawson ND, Kulik M, Childs S, Lekven AC, Garrity DM, Moon RT, Fishman MC, Lechleider RJ, Weinstein BM. Disruption of acvrl1 increases endothelial cell number in zebrafish cranial vessels. *Development* **129**: 3009-3019, 2002.

124. Saharinen P, Helotera H, Miettinen J, Norrmen C, D'Amico G, Jeltsch M, Langenberg T, Vandevelde W, Ny A, Dewerchin M, Carmeliet P, Alitalo K. Claudin-like protein 24 interacts with the VEGFR-2 and VEGFR-3 pathways and regulates lymphatic vessel development. *Genes & development* **24**: 875-880, 2010.

125. Saif WS, Knost JA, Chiorean G, Kambhampati SRP, Yu D, Pytowski B, Qin A, Kauh JS, O'Neil BH. Phase I study of anti-VEGF receptor-3 (VEGFR-3) monoclonal antibody (mab) LY3022856/IMC-3C5 (3C5). *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **33**: (suppl; abstr 3530), 2015.

126. Sanderson LE, Chien AT, Astin JW, Crosier KE, Crosier PS, Hall CJ. An inducible transgene reports activation of macrophages in live zebrafish larvae. *Developmental and comparative immunology* **53**: 63-69, 2015.

127. Santoro MM. Antiangiogenic cancer drug using the zebrafish model. *Arteriosclerosis, thrombosis, and vascular biology* **34**: 1846-1853, 2014.

128. Santoro MM, Pesce G, Stainier DY. Characterization of vascular mural cells during zebrafish development. *Mechanisms of development* **126**: 638-649, 2009.

129. Schuermann A, Helker CS, Herzog W. Angiogenesis in zebrafish. *Seminars in cell & developmental biology* **31**: 106-114, 2014.

130. Shojaei F. Anti-angiogenesis therapy in cancer: current challenges and future perspectives. *Cancer letters* **320**: 130-137, 2012.

131. Siekmann AF, Lawson ND. Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. *Nature* **445**: 781-784, 2007.

132. Stacker SA, Williams RA, Achen MG. Lymphangiogenic growth factors as markers of tumor metastasis. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica* **112**: 539-549, 2004.

133. Stacker SA, Williams SP, Karnezis T, Shayan R, Fox SB, Achen MG. Lymphangiogenesis and lymphatic vessel remodelling in cancer. *Nature reviews. Cancer* **14**: 159-172, 2014.

134. Stainier DY, Weinstein BM, Detrich HW, 3rd, Zon LI, Fishman MC. Cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. *Development* **121**: 3141-3150, 1995.

135. Stoletov K, Kato H, Zardouzian E, Kelber J, Yang J, Shattil S, Klemke R. Visualizing extravasation dynamics of metastatic tumor cells. *Journal of cell science* **123**: 2332-2341, 2010.

136. Stoletov K, Montel V, Lester RD, Gonias SL, Klemke R. High-resolution imaging of the dynamic tumor cell vascular interface in transparent zebrafish. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 17406-17411, 2007.

137. Sumoy L, Keasey JB, Dittman TD, Kimelman D. A role for notochord in axial vascular development revealed by analysis of phenotype and the expression of VEGR-2 in zebrafish flh and ntl mutant embryos. *Mechanisms of development* **63**: 15-27, 1997.

138. Sun XY, Zheng YP, Lin DH, Zhang H, Zhao F, Yuan CS. Potential anti-cancer activities of Furanodiene, a Sesquiterpene from Curcuma wenyujin. *The American journal of Chinese medicine* **37**: 589-596, 2009.

139. Tang Q, Abdelfattah NS, Blackburn JS, Moore JC, Martinez SA, Moore FE, Lobbardi R, Tenente IM, Ignatius MS, Berman JN, Liwski RS, Houvras Y, Langenau DM. Optimized cell transplantation using adult rag2 mutant zebrafish. *Nature methods* **11**: 821-824, 2014.

140. Tang Q, Moore JC, Ignatius MS, Tenente IM, Hayes MN, Garcia EG, Torres Yordan N, Bourque C, He S, Blackburn JS, Look AT, Houvras Y, Langenau DM. Imaging tumour cell heterogeneity following cell transplantation into optically clear immune-deficient zebrafish. *Nature communications* **7**: 10358, 2016.

141. Tao S, Witte M, Bryson-Richardson RJ, Currie PD, Hogan BM, Schulte-Merker S. Zebrafish prox1b mutants develop a lymphatic vasculature, and prox1b does not specifically mark lymphatic endothelial cells. *PloS one* **6**: e28934, 2011.

142. Thompson MA, Ransom DG, Pratt SJ, MacLennan H, Kieran MW, Detrich HW, 3rd, Vail B, Huber TL, Paw B, Brownlie AJ, Oates AC, Fritz A, Gates MA, Amores A, Bahary N, Talbot WS, Her H, Beier DR, Postlethwait JH, Zon LI. The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. *Developmental biology* **197**: 248-269, 1998.

143. Torres-Vazquez J, Gitler AD, Fraser SD, Berk JD, Van NP, Fishman MC, Childs S, Epstein JA, Weinstein BM. Semaphorin-plexin signaling guides patterning of the developing vasculature. *Developmental cell* **7**: 117-123, 2004.

144. Tran TC, Sneed B, Haider J, Blavo D, White A, Aiyejorun T, Baranowski TC, Rubinstein AL, Doan TN, Dingledine R, Sandberg EM. Automated, quantitative screening assay for antiangiogenic compounds using transgenic zebrafish. *Cancer research* **67**: 11386-11392, 2007.

145. Trovik CS, Bauer HC, Alvegard TA, Anderson H, Blomqvist C, Berlin O, Gustafson P, Saeter G, Walloe A. Surgical margins, local recurrence and metastasis in soft tissue sarcomas: 559 surgically-treated patients from the Scandinavian Sarcoma Group Register. *European journal of cancer* **36**: 710-716, 2000.

146. van Impel A, Zhao Z, Hermkens DM, Roukens MG, Fischer JC, Peterson-Maduro J, Duckers H, Ober EA, Ingham PW, Schulte-Merker S. Divergence of zebrafish and mouse lymphatic cell fate specification pathways. *Development* **141**: 1228-1238, 2014.

147. Veinotte CJ, Dellaire G, Berman JN. Hooking the big one: the potential of zebrafish xenotransplantation to reform cancer drug screening in the genomic era. *Disease models & mechanisms* **7**: 745-754, 2014.

148. Vitale G, Gaudenzi G, Dicitore A, Cotelli F, Ferone D, Persani L. Zebrafish as an innovative model for neuroendocrine tumors. *Endocrine-related cancer* **21**: R67-83, 2014.

149. Vlecken DH, Bagowski CP. LIMK1 and LIMK2 are important for metastatic behavior and tumor cell-induced angiogenesis of pancreatic cancer cells. *Zebrafish* **6**: 433-439, 2009.

150. Walton EM, Cronan MR, Beerman RW, Tobin DM. The Macrophage-Specific Promoter mfap4 Allows Live, Long-Term Analysis of Macrophage Behavior during Mycobacterial Infection in Zebrafish. *PloS one* **10**: e0138949, 2015.

151. Wang C, Tao W, Wang Y, Bikow J, Lu B, Keating A, Verma S, Parker TG, Han R, Wen XY. Rosuvastatin, identified from a zebrafish chemical genetic screen for antiangiogenic compounds, suppresses the growth of prostate cancer. *European urology* **58**: 418-426, 2010.

152. Wang J, Cao Z, Zhang XM, Nakamura M, Sun M, Hartman J, Harris RA, Sun Y, Cao Y. Novel mechanism of macrophage-mediated metastasis revealed in a zebrafish model of tumor development. *Cancer research* **75**: 306-315, 2015.

153. Wang Y, Wang B, Guerram M, Sun L, Shi W, Tian C, Zhu X, Jiang Z, Zhang L. Deoxypodophyllotoxin suppresses tumor vasculature in HUVECs by promoting cytoskeleton

remodeling through LKB1-AMPK dependent Rho A activatio. *Oncotarget* **6**: 29497-29512, 2015.

154. Weinstein BM, Stemple DL, Driever W, Fishman MC. Gridlock, a localized heritable vascular patterning defect in the zebrafish. *Nature medicine* **1**: 1143-1147, 1995.

155. White RM, Sessa A, Burke C, Bowman T, LeBlanc J, Ceol C, Bourque C, Dovey M, Goessling W, Burns CE, Zon LI. Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell stem cell* **2**: 183-189, 2008.

156. Wiens KM, Lee HL, Shimada H, Metcalf AE, Chao MY, Lien CL. Platelet-derived growth factor receptor beta is critical for zebrafish intersegmental vessel formation. *PloS one* **5**: e11324, 2010.

157. Wittamer V, Bertrand JY, Gutschow PW, Traver D. Characterization of the mononuclear phagocyte system in zebrafish. *Blood* **117**: 7126-7135, 2011.

158. Wu Q, Deng S, Li L, Sun L, Yang X, Liu X, Liu L, Qian Z, Wei Y, Gong C. Biodegradable polymeric micelle-encapsulated quercetin suppresses tumor growth and metastasis in both transgenic zebrafish and mouse models. *Nanoscale* **5**: 12480-12493, 2013.

159. Xia Y, Song X, Li D, Ye T, Xu Y, Lin H, Meng N, Li G, Deng S, Zhang S, Liu L, Zhu Y, Zeng J, Lei Q, Pan Y, Wei Y, Zhao Y, Yu L. YLT192, a novel, orally active bioavailable inhibitor of VEGFR2 signaling with potent antiangiogenic activity and antitumor efficacy in preclinical models. *Scientific reports* **4**: 6031, 2014.

160. Yang JH, Hu J, Wan L, Chen LJ. Barbigerone inhibits tumor angiogenesis, growth and metastasis in melanoma. *Asian Pacific journal of cancer prevention : APJCP* **15**: 167-174, 2014.

161. Yang X, Cui W, Yu S, Xu C, Chen G, Gu A, Li T, Cui Y, Zhang X, Bian X. A synthetic dl-nordihydroguaiaretic acid (Nordy), inhibits angiogenesis, invasion and proliferation of glioma stem cells within a zebrafish xenotransplantation model. *PloS one* **9**: e85759, 2014.

162. Yang XJ, Cui W, Gu A, Xu C, Yu SC, Li TT, Cui YH, Zhang X, Bian XW. A novel zebrafish xenotransplantation model for study of glioma stem cell invasion. *PloS one* **8**: e61801, 2013.

163. Yaniv K, Isogai S, Castranova D, Dye L, Hitomi J, Weinstein BM. Live imaging of lymphatic development in the zebrafish. *Nature medicine* **12**: 711-716, 2006.

164. Zakrzewski JL, Tuckett AZ, van den Brink MR. Transforming lymph nodes into tissue factories. *Nature biotechnology* **30**: 958-959, 2012.

165. Zhang S, Cao Z, Tian H, Shen G, Ma Y, Xie H, Liu Y, Zhao C, Deng S, Yang Y, Zheng R, Li W, Zhang N, Liu S, Wang W, Dai L, Shi S, Cheng L, Pan Y, Feng S, Zhao X, Deng H, Yang S, Wei Y. SKLB1002, a novel potent inhibitor of VEGF receptor 2 signaling, inhibits angiogenesis and tumor growth in vivo. *Clinical cancer research : an official journal of the American Association for Cancer Research* **17**: 4439-4450, 2011.

166. Zhao C, Wang X, Zhao Y, Li Z, Lin S, Wei Y, Yang H. A novel xenograft model in zebrafish for high-resolution investigating dynamics of neovascularization in tumors. *PloS one* **6**: e21768, 2011.

167. Zhao D, Qin C, Fan X, Li Y, Gu B. Inhibitory effects of quercetin on angiogenesis in larval zebrafish and human umbilical vein endothelial cells. *European journal of pharmacology* **723**: 360-367, 2014.

168. Zheng MW, Zhang CH, Chen K, Huang M, Li YP, Lin WT, Zhang RJ, Zhong L, Xiang R, Li LL, Liu XY, Wei YQ, Yang SY. Preclinical evaluation of a novel orally available SRC/Raf/VEGFR2 inhibitor, SKLB646, in the treatment of triple negative breast cancer. *Molecular cancer therapeutics* **15**: 366-378, 2015.

169. Zhong L, Fu XY, Zou C, Yang LL, Zhou S, Yang J, Tang Y, Cheng C, Li LL, Xiang R, Chen LJ, Chen YZ, Wei YQ, Yang SY. A preclinical evaluation of a novel multikinase inhibitor, SKLB-329, as a therapeutic agent against hepatocellular carcinoma. *International journal of cancer. Journal international du cancer* **135**: 2972-2983, 2014.

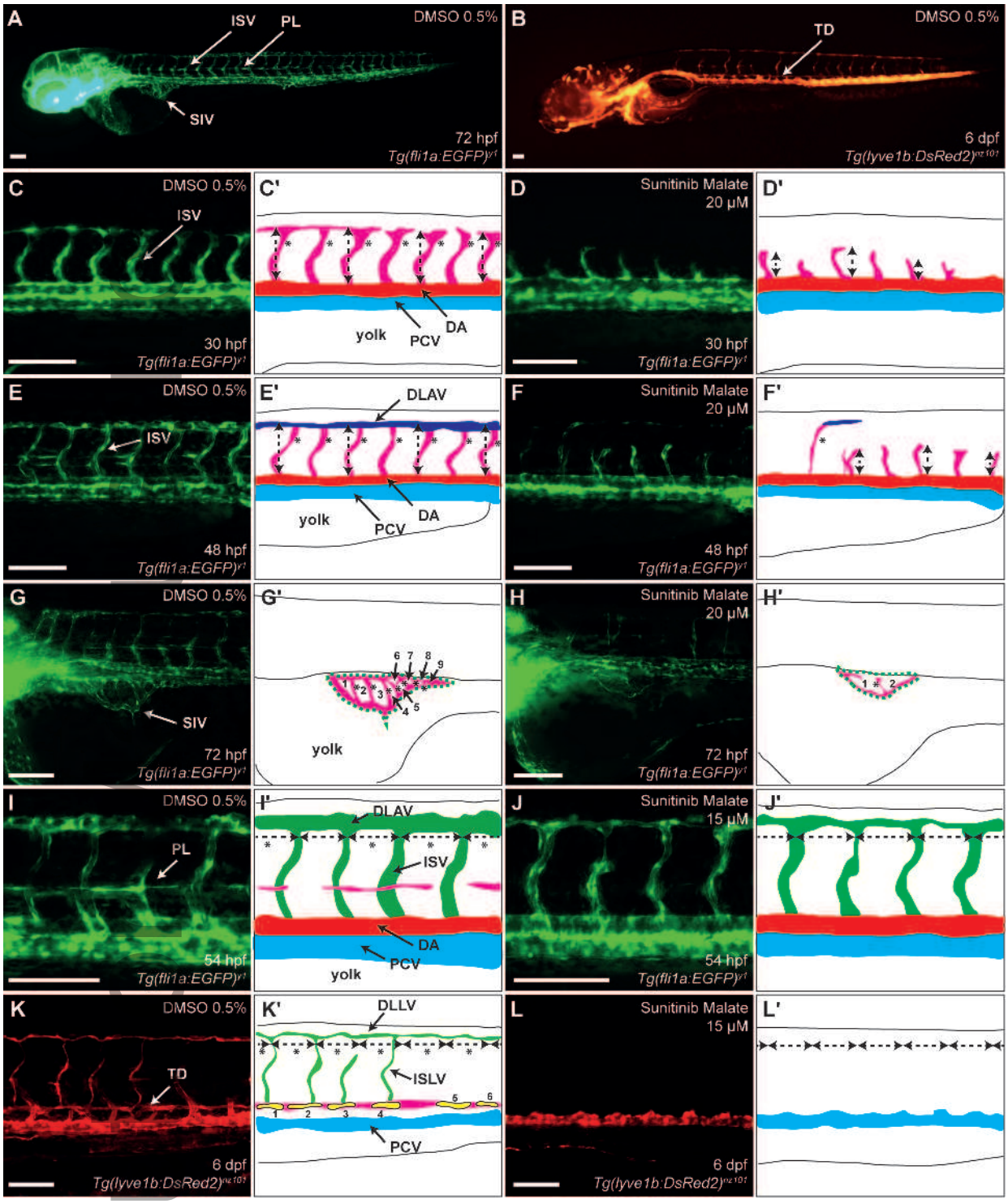
170. Zhong ZF, Hoi PM, Wu GS, Xu ZT, Tan W, Chen XP, Cui L, Wu T, Wang YT. Anti-angiogenic effect of furanodiene on HUVECs in vitro and on zebrafish in vivo. *Journal of ethnopharmacology* **141**: 721-727, 2012.

171. Zhou Z, Zhao C, Wang L, Cao X, Li J, Huang R, Lao Q, Yu H, Li Y, Du H, Qu L, Shou C. A VEGFR1 antagonistic peptide inhibits tumor growth and metastasis through VEGFR1-PI3K-AKT signaling pathway inhibition. *American journal of cancer research* **5**: 3149-3161, 2015.

172. Zhu F, Hall CJ, Crosier PS, Wlodkowic D. Interfacing Lab-on-a-Chip Embryo Technology with High-Definition Imaging Cytometry. *Zebrafish* **12**: 315-318, 2015.

173. Zhu XY, Xia B, Liu HC, Xu YQ, Huang CJ, Gao JM, Dong QX, Li CQ. Closantel Suppresses Angiogenesis and Cancer Growth in Zebrafish Models. *Assay and drug development technologies*, 2016.

174. Zygmunt T, Gay CM, Blondelle J, Singh MK, Flaherty KM, Means PC, Herwig L, Krudewig A, Belting HG, Affolter M, Epstein JA, Torres-Vazquez J. Semaphorin-PlexinD1 signaling limits angiogenic potential via the VEGF decoy receptor sFlt1. *Developmental cell* **21**: 301-314, 2011.



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