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Modeling Tumor Angiogenesis with Zebrafish

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1. Introduction

Angiogenesis is process by which new blood vessels arise from endothelial cells in the existing vessels. In normal circumstances, the initiation, formation, maturation, remodeling and regression of endothelial cells in this process are strictly regulated. During tumor formation, the regulation of angiogenesis is disrupted and endothelial remodeling and regression are usually absent. Therefore, study on angiogenesis is of important relevance to cancer biology and therapeutic intervention (Carmeliet & Jain, 2000), especially in cancers where tumor growth depends on extensive vascularization (Folkman, 2002).

A number of in vitro and in vivo models have been used for the study of angiogenesis. These include an endothelial cell line derived from human umbilical cord vein endothelial cells (HUVEC) (Jaffe et al., 1973) as well as a number of organ specific endothelial cell lines. With these cell lines, endothelial cell proliferation, differentiation and migration have been characterized. However, information about how endothelial cells interact with their neighboring cells is often lacking. In this regards, explant cultures (Brown et al., 1996; Jung et al., 2001) might be more representative of the complex interaction between endothelial and the supporting cells. Nevertheless, the issues of incomplete microenvironment, animal to animal variability and technical difficulties from relatively time-consuming and labor-intensive tissue isolation and culture might limit the application of these models.

In vivo models of angiogenesis have also been developed using chick embryo, rabbit and mouse (reviewed by Staton et al., 2009). They provide a more accurate physiological model of angiogenesis and when implanted with primary tumors or cancer cell lines, they can also provide important mechanistic insights to tumor angiogenesis. However, large-scale chemical screening with these models is difficult due to the cost and space needed for husbandry facilities.

Zebrafish has emerged as a model organism for the study of genetics and human diseases. Compare with other vertebrate models, this small tropical fish offers distinctive advantages. Firstly, zebrafish embryos are externally fertilized and optically transparent, allowing direct visualization during embryonic development. Secondly, these embryos are amenable to reverse genetic manipulation including gene knock-down, over-expression or transgenesis by microinjection. Thirdly, the high fecundity of zebrafish enables adequate experimental duplicates and facilitates high through-put forward genetic screening. Mating a single pair of adult zebrafish can produce hundreds of eggs in one day. Fourthly, stable tissue-specific transgenic fish-lines are available, allowing direct visualization of various developmental processes. Lastly, husbandry and maintenance of zebrafish colonies are space and cost effective.

Early zebrafish embryonic vascular development begins at around 12 hour-post-fertilization (hpf) when hemangioblasts first exist along the lateral plate mesoderm. Later at around 24 hpf, the development of dorsal aorta (DA) and dorsal vein (DV), forming the first circulation loop. Subsequently, angiogenesis including the development of inter-segmental vessels (ISV) and sub-intestinal veins (SIV) occurs. Important growth factors and associated receptor tyrosine kinases as well as Notch signaling pathway regulating mammalian vascular development are conserved in zebrafish (Liang et al., 1998; Habeck et al., 2002; Goishi and Klagsbrun, 2004; Siekmann and Lawson ND, 2007). Here, we explore the potential of using zebrafish *in vivo* to model and more importantly to screen potential therapeutic agents targeting tumor angiogenesis.

2. Zebrafish embryonic angiogenesis

During zebrafish embryonic development, angiogenesis is characterized by the sprouting of inter-segmental vessels in the trunk between each somite initiated around 24 hpf as well as the development of sub-intestinal veins initiated around 48 hpf (Isogai et al., 2001; Lawson and Weinstein, 2002a). Although some argued the sprouting of ISV would represent type II vasculogenesis (Childs et al., 2002), these two processes are well accepted to represent early embryonic angiogenesis.

Traditional assay to examine zebrafish angiogenesis includes alkaline-phosphatase (AP) staining of endothelial cells and whole-mount *in situ* hybridization of genes associated with vascular development such as *fli1*, *flk1*, *flt4*, *efnb2a* etc. Although *in situ* hybridization could provide more specific information such as artery or vein specification (Lawson and Weinstein, 2002a), these methods preclude direct and real-time visualization of the vasculature. Also, it takes days to complete staining protocols. These shortcomings have limited the application of zebrafish model until the recent advancement in zebrafish transgenesis and the availability of tissue-specific stable fluorescent reporter transgenic lines. With the use of fluorescent report transgenic zebrafish line such as *Tg(fli1:egfp)* (Lawson and Weinstein, 2002b) or *Tg(flkl:egfp)* (Jin et al., 2005), embryonic angiogenesis could be easily monitored real-time under fluorescent microscope. Figure 1 demonstrates the development of ISV and SIV at 48 and 72 hpf with *Tg(flkl:egfp)* and *Tg(fli1:egfp)*.

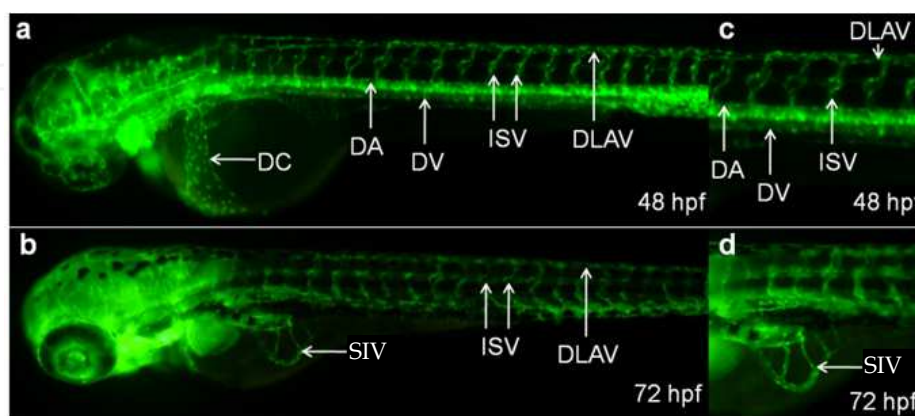


Fig. 1. Endothelial fluorescent transgenic zebrafish embryos showing vascular development at (a, c) 48 and (b, d) 72 hpf. (a, c): *Tg(flkl:egfp)*; (b, d): *Tg(fli1:egfp)*. DC: Duct of Curvier; DA: Dorsal aorta; DLAV: dorsal longitudinal anastomotic vessels; DV: Dorsal vein; ISV: Inter-segmental vessel; SIV: sub-intestinal vessels.

3. Modeling tumor angiogenesis in zebrafish

3.1 Gene regulation of zebrafish angiogenesis

While angiogenesis is important for tumor growth and metastasis (Folkman, 2002), the precise mechanism and regulation of tumor angiogenesis remains unclear. Therefore, understanding angiogenesis during normal embryonic development might provide insight into how this process would be perturbed during tumor growth. Previous studies have demonstrated that genes that are involved in tumor angiogenesis such as *galectin-1* (Thijssen et al., 2006), *CXCR7* (Miao et al., 2007), *angiomodulin* (Hooper et al., 2009) and *PDGFR- β /B-Raf* (Murphy et al., 2010) may also play a role in embryonic angiogenesis. The zebrafish is unique in this respect because the circulatory system is dispensable during the first few days of embryonic development, enabling study of genes by specific knock-down that is otherwise lethal in the mammalian system.

3.2 Survivin and zebrafish angiogenesis

We have previously identified zebrafish survivin-1 (Ma et al., 2007a) as an important regulator of embryonic angiogenesis. Survivin exerts its effect through anti-apoptosis and interaction with VEGF receptor kinase pathway. Survivin is the smallest member of the inhibitor of apoptosis (IAP) gene family with a single Baculovirus IAP Repeat (BIR) domain and an extended -COOH terminal α -helical coiled coil (Altieri, 2004). While it is not expressed in most normal adult tissues, survivin is highly expressed in solid and hematological malignancies, where it has been linked to tumor angiogenesis and represented a potential target for anti-cancer therapy (Graaf et al., 1998; Altieri, 2003). During human and murine embryonic development, survivin is ubiquitously expressed (Adida et al., 1998). However, homozygous knock-out of *survivin* in mouse ES cells results in disrupted microtubule formation and polyploidy as well as early embryonic fatality, precluding characterization of its functions during murine development (Uren et al., 2000) and therefore zebrafish embryo was considered an alternative embryonic model.

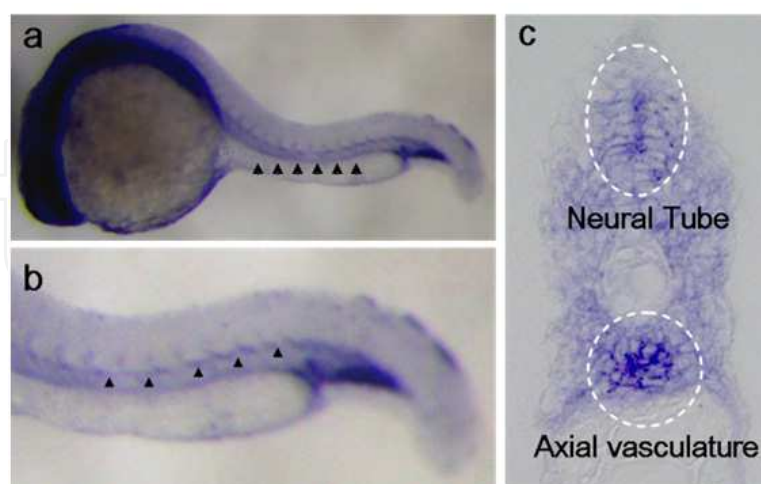


Fig. 2. Expression of *survivin-1* in zebrafish embryo as shown by ISH at 26 hpf. (a,b) Lateral view of whole-mount ISH and black arrowheads denote expression along axial vasculatures. (c) Transverse section of whole-mount ISH showing expression at neural tube and axial vasculatures. Adopted and modified from figure originally published in Ma et al 2007a (with permission).

In zebrafish embryos, *survivin* gene is duplicated into *survivin-1* and *survivin-2*. During embryonic development, *survivin-1* and *survivin-2* are differentially expressed with distinctive functions in the vasculature and hematopoietic tissues (Ma et al., 2007a; Ma et al., 2009). Both *survivin-1* and *survivin-2* share a highly homologous functional BIR-domain and similar functions at cellular level. Therefore, the distinctive roles of *survivin-1* and *survivin-2* during embryonic development may be related to a large extent to their difference in spatial expression (Ma et al., 2009). In particular, *survivin-1* predominantly expressed along the neural tube and axial vasculature at 26 hpf (Figure 2). Knock-down of *survivin-1* with anti-sense morpholino gives rise to defective angiogenesis as shown by defective sprouting of ISV as well as SIV (Figure 3). Vasculogenesis, demonstrated by the formation of axial vasculatures, was not affected.

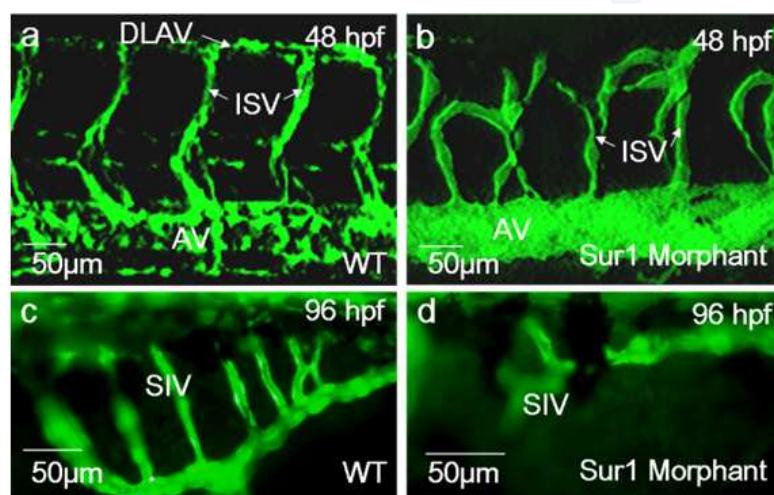


Fig. 3. Effect of *survivin-1* knock-down on zebrafish embryonic angiogenesis. (a, b): Confocal microscopy of *Tg(fli1:egfp)* embryos at 48 hpf either (a) uninjected or (b) injected with *survivin-1* morpholino (MO). Noted the defective sprouting of ISV and the failure to form dorsal longitudinal anastomotic vessels (DLAV) in *survivin-1* (Sur1) morphant. AV: Axial vasculatures. (c, d): *Tg(fli1:egfp)* embryos at 96 hpf showing failure to develop the SIV in Sur1 morphant. Adopted and modified from figure originally published in Ma et al 2007a (with permission).

In vitro and tumorigenesis studies have shown that survivin mediates the angiogenic effects of VEGF (Tran et al., 1999; Mesri et al., 2001; Beierle et al., 2005). In zebrafish embryos, VEGF signaling is also important for angiogenesis. The *schwentine* mutant with defective VEGFR tyrosine kinase, *flk1* (Habeck et al., 2002) has perturbed angiogenesis. In addition, phospholipase C- γ (*plc- γ*) mutant (*y10*) (Lawson et al., 2003) as well as knock-down morphant (Ma et al., 2007b) also exhibit specific defects in angiogenesis. VEGF induces ectopic angiogenesis and up-regulates *survivin-1* mRNA expression (Figure 4a-c), suggesting that *survivin-1* may mediate the angiogenic effect of VEGF. For instance, we only detect modest apoptotic TUNEL staining in the axial vasculature of *survivin-1* morphant (Figure 4d, e) but not a direct causal link between increased apoptosis and the angiogenesis defect. VEGF might prevent apoptosis (Gupta et al., 1999) and VEGF inhibitors exert pro-apoptotic effect on endothelial cells (reviewed by Epstein, 2007). While apoptotic signal was readily detected along the neural tube of *survivin-1* morphant (Figure 4d, e), *survivin-1* might exert its anti-apoptotic effect in a non-cell autonomous fashion downstream of VEGF, regulating the

signaling cues for angioblasts to migrate from aorta to the dorsal aspect of the neural tube and to the inter-phase between notochord and the somites before ISV sprouting (Childs et al., 2002).

3.3 Zebrafish xenograft model of tumor angiogenesis

Recently, zebrafish xenograft models have been developed through xenotransplantation of human primary tumor cells or cancer cell lines into yolk sac of 48 hpf zebrafish embryos (Lee LM et al., 2005; Haldi et al., 2006; Topczewska et al., 2006; Nicoli et al., 2007; Marques et al., 2009). Without a functional immune system at this early embryonic stage, immunosuppression is not needed. The experimental procedures of transplanting fluorescent labeled human cancer cells into perivitelline space of 48 hpf zebrafish embryos was subsequently published (Nocoli and Presta, 2007). In these models, cancer cells were shown to be engrafted into the yolk sac with proliferation and migration. More importantly, angiogenesis were induced in SIV with infiltration of blood vessels into the cancer mass. Combining with fluorescent reporter transgenic lines, these models serve as a promising platform to study the biology of tumor angiogenesis and its microenvironment including hypoxia (Lee SL et al., 2009) and LIM domain kinase 1 and 2 (Vlecken and Bagowski, 2009).

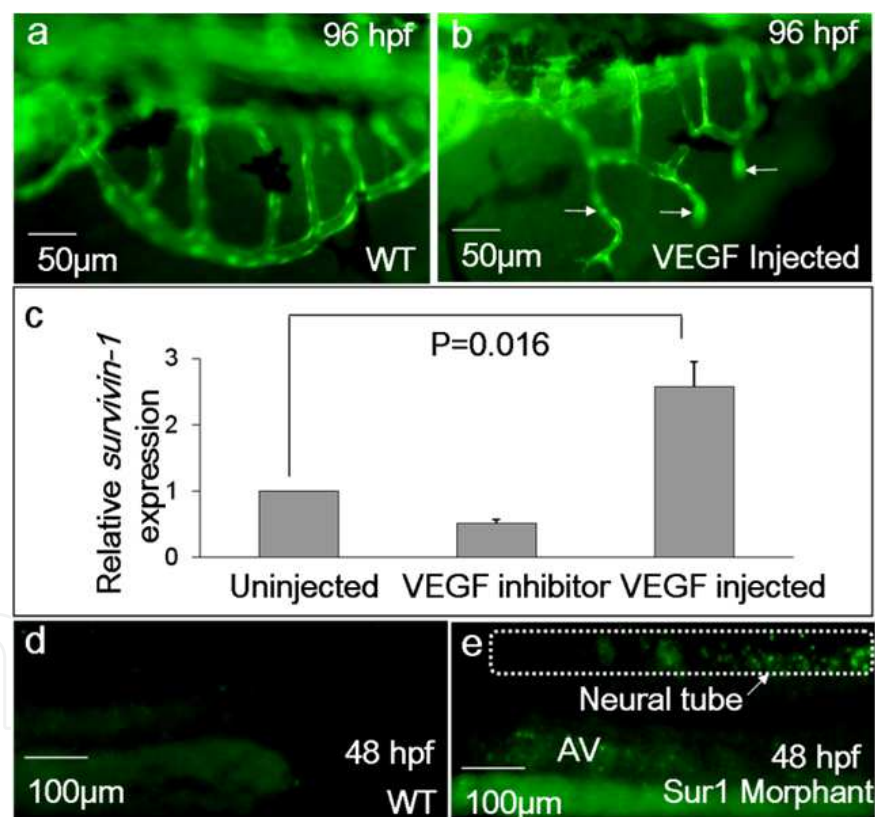


Fig. 4. Survivin-1 interact with VEGF signaling and exert anti-apoptotic activity during zebrafish embryonic angiogenesis. (a, b): Microscopy of *Tg(fli1:egfp)* embryos at 96 hpf either (a) uninjected or (b) injected with human VEGF (2 ng) protein, which induces ectopic angiogenesis (white arrows). (c): relative expression of *survivin-1* mRNA measured by quantitative RT-PCR. (d, e): Whole-mount TUNEL assay in embryos injected with either (e) random sequence or (b) *Sur1* MO, which shows positive staining in the area of developing neural tube and at the vicinity of the axial vasculatures (AV) in *Sur1* morphant. Adopted and modified from figure originally published in Ma et al 2007a (with permission).

4. Screening potential therapeutic agents with zebrafish embryos

4.1 Large-scale chemical screening platform

Since angiogenesis is crucial for tumor growth and progression, anti-angiogenic agents have been investigated as potential anti-cancer therapies (Demetri et al., 2002; Cunningham et al., 2004; Shepherd et al., 2005; Van et., al 2007; Hudes et al., 2007). Chemical screening based on in vivo tumor xenograft models are often limited by the relatively low throughput and long read-out time. In this respect, the zebrafish embryo is uniquely suitable for large-scale chemical screening because of the advantages aforementioned. In particular, using the Tg(*flk1:egfp*) or Tg(*fli1:egfp*) embryos, one could conduct large-scale in vivo screening against chemical libraries in a cost-effective way. To examine their effects on the initiation and regression of angiogenesis, embryos will be exposed to chemicals at different concentrations and developmental stages, either before angiogenesis (12 hpf), or after sprouting of ISV and development of SIV (48 hpf). Chemicals that specifically inhibit ISV and SIV formation after 12 hpf likely inhibit the initiation of angiogenesis and those that affect ISV and SIV after their formation at 48 hpf likely induce vascular regression (Figure 5).

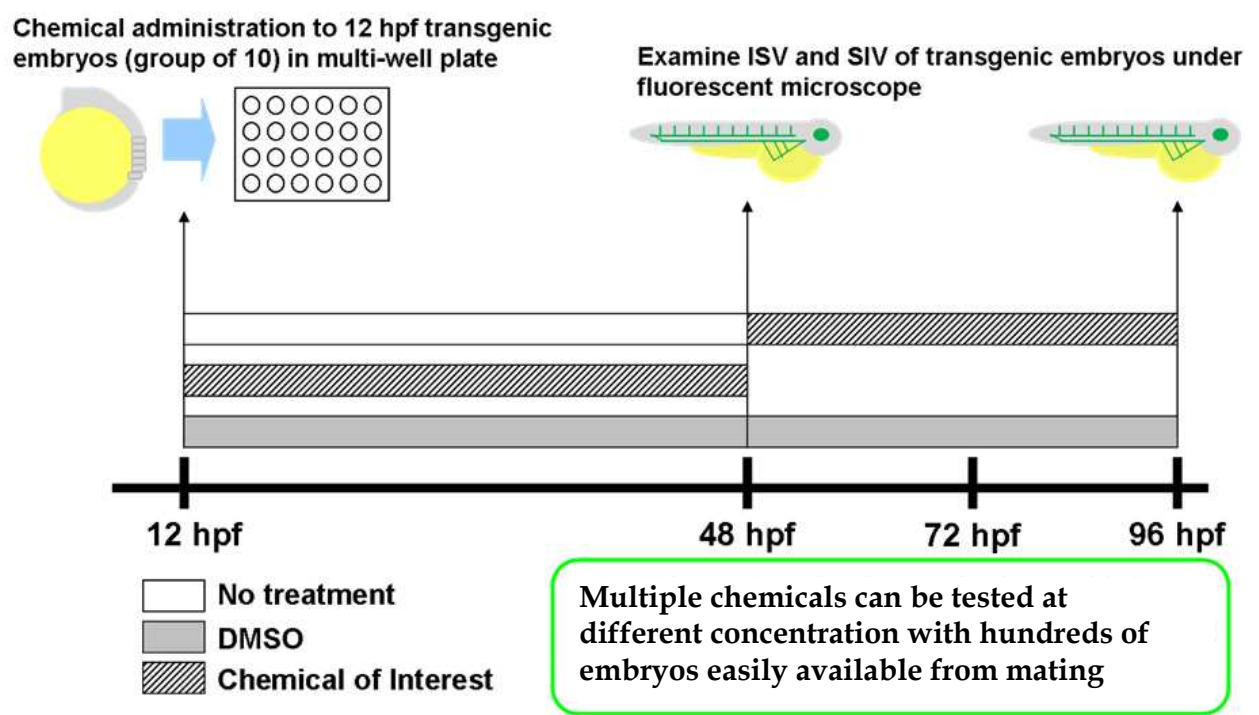


Fig. 5. Cost-effective anti-angiogenic chemicals screening platform with zebrafish embryos.

Both anti-angiogenic mechanisms are considered important component in cancer therapy. This protocol may enable identification of potential anti-angiogenic compounds at high throughput and provide us with novel information about the link between embryonic and tumor angiogenesis. Figure 6 shows the use of Tg(*flk1:egfp*) embryos as a platform to demonstrate anti-angiogenic activity of VEGFR tyrosine kinase inhibitor and anti-cancer drugs (multi-kinase inhibitors) sorafenib and sunitinib.

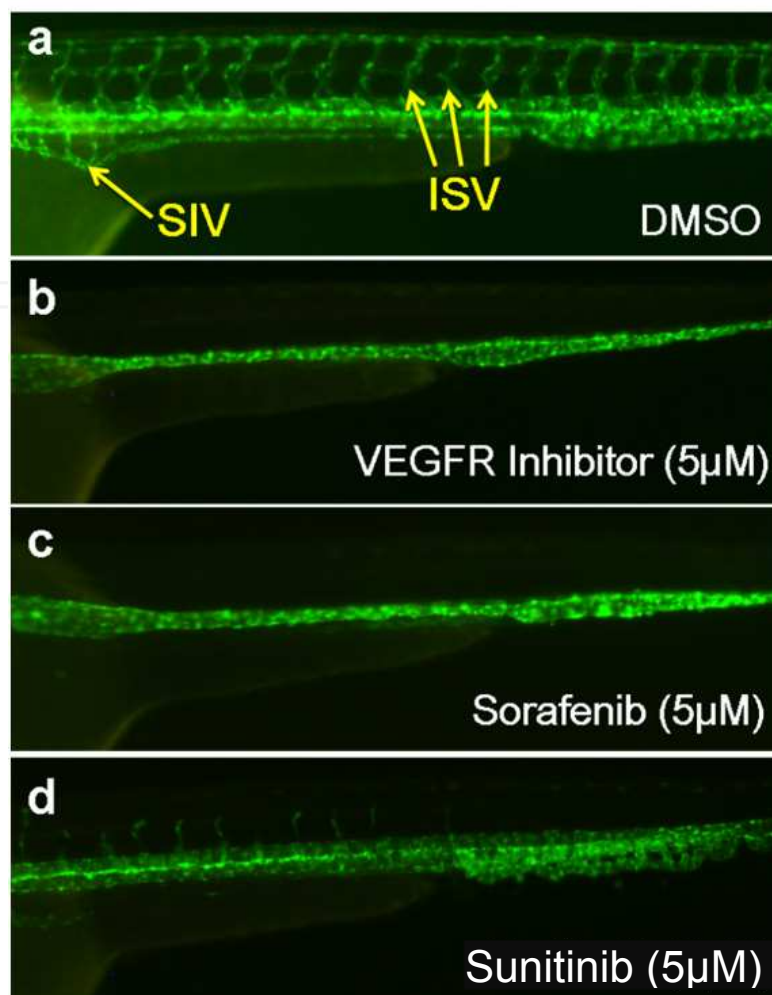


Fig. 6. Demonstration of anti-angiogenic effect of kinase inhibitors with transgenic zebrafish embryos. Microscopy of Tg(*flk1:egfp*) embryos at 48 hpf treated with (a) DMSO, (b) VEGFR tyrosine kinase inhibitor, (c) sorafenib and (d) sunitinib. Treatment with these inhibitors significantly perturbed zebrafish embryonic angiogenesis as shown by development of ISV and SIV.

5. Conclusion

Since angiogenesis is crucial for tumor growth and progression, it may present a potential target for cancer therapy. A number of anti-angiogenic agents targeting at the VEGF signaling pathway are being evaluated and large-scale chemical screening is needed to provide more candidates that can be tested in clinical trials. In this respect, the zebrafish embryos have emerged as a promising model that can shed important lights to the biology of physiological and tumor angiogenesis at whole organism level and allow cost-effective high throughput chemical screening. A number of new genetic modification technologies are now available that can specifically interrogate gene function related to angiogenesis. For instance, artificial endonucleases constructed by fusing non-specific nuclease domain with specific DNA binding domains (Egger, 2008; Foley et al., 2009a; Foley et al., 2009b; Miller et al., 2011; Cermak et al., 2011; Sander et al., 2011) can now be used to target specific genes from zebrafish genome. An in vivo protein trap mutagenesis system (Clark et al., 2011) is

also available that can simultaneously reveal spatio-temporal protein expression dynamics and assess gene function in zebrafish embryos. These new technologies greatly improve the efficiency of zebrafish genetic modifications and forward genetic screening, making zebrafish a more powerful model organism for angiogenesis.

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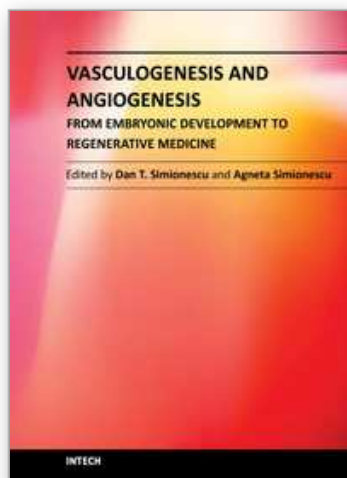
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Vasculogenesis and Angiogenesis - from Embryonic Development to Regenerative Medicine

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Vasculogenesis is the process of new blood vessel formation during embryonic development of the cardiovascular system. This is followed by formation of a vascular tree and finally the cardiovascular system with the myriad of blood vessels that nourish all tissues and organs. Angiogenesis, on the other hand is the process by which new blood vessels take shape from existing blood vessels by "sprouting" of endothelial cells thus expanding the vascular tree. Both scenarios are based on activation, migration, proliferation and maturation of unique precursor cells. The study of blood vessel formation is an essential component of embryonic development, congenital malformations, degenerative diseases, inflammation and cancer and thus has widespread appeal to the biomedical field. Moreover, scientists are now harnessing this information for the purpose of building living blood vessel substitutes for replacement of diseased arteries and veins. This book highlights novel advances in the field of vasculogenesis and angiogenesis, including embryogenesis and development, regulation of progenitor cells, cancer and blood vessel regeneration. We consider this book a good initial source of information for graduate students, medical students and scientists interested in the intricacies of blood vessel formation, maturation, disease and replacement.

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