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SYMPOSIUM REVIEW

Fishing for novel angiogenic therapies

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The zebrafish has recently emerged as an important model for the study of vascular embryogenesis. Its genetic accessibility, external development, and optically clear embryo are just a few of the features that set the zebrafish apart as a particularly well-suited model for studying vascular development. However, there is little precedent for its use as a tool for the experimental study of therapeutic angiogenesis. Here, we review the use of the zebrafish for studying vascular development and patterning, and discuss how the zebrafish might be used more directly as a model for developing and testing effective therapeutic angiogenesis approaches.
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Abbreviations: EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; A-V, arterial venous; vegf, vascular endothelial growth factor; pleg 1, phospholipase C gamma-1; shh, sonic hedgehog

Introduction

Understanding the mechanisms of blood vessel formation has been a problem of interest to basic scientists for many years, but the clinical importance of these sort of studies and the potential therapeutic applications of regulating angiogenesis have only recently come to the forefront. Therapeutic angiogenesis initially emerged as an approach to the treatment of disease through the induction of new blood vessel formation within ischemic tissues for tissue repair and/or regeneration. It has since come to include the inhibition of blood vessel formation in pathological processes dependent on angiogenesis, in particular tumor growth. The identification of specific antiangiogenic factors capable of inhibiting tumor progression in animal models has led to significant investment of resources and advancement in this area of research. Although a number of clinical trials involving both pro- and antiangiogenic therapies have been initiated, most have not attained their anticipated success (Schwarz *et al*., 2000; Satchi-Fainaro, 2002). This has led to the realization that regulation of the angiogenic process is perhaps more complex than originally hypothesized, requiring more than a simple shift in the balance between angiogenic stimulators and inhibitors (Giordano & Johnson, 2001). The emergence of tissue engineering as a *bona fide* research endeavor has also created the additional need to better understand the mechanisms of development and diversification of the vasculature. The success of organ and tissue regeneration depends on the ability to establish a vascular supply within different tissues (reviewed in Moldovan & Ferrari, 2002; Polverini, 2002), and it has become clear that the vascular beds of various organs and tissues differ from one another and that these differences are likely to be important for the proper function of the vessels in these organs (Kolonin *et al*., 2001; Arap *et al*., 2002). Among other things, tissues differ in vessel-type distribution, that is, the relative percentage of arteries, venules, and capillaries. Tissues such as the epicardial surface have a high percentage of drainage venules, while adipose tissues have a high percentage of capillaries. Biomedical implants on these surfaces do not elicit formation of the native vessel-type distribution (Kellar *et al*., 2002). For tissue engineering to generate both biological and synthetic replacement tissues successfully, the ability to generate a tissue-specific vascular bed must coexist.

Potential clinical applications have prompted renewed interest in understanding the process of angiogenesis and its regulation, but attaining this understanding requires identification and use of models for the study of vascular development and regulation that are truly representative of the *in vivo* complexity of the vasculature. While many *in vitro* systems facilitate ascertaining specific information regarding endothelial cell biology, they are not able to recreate the microenvironment of an intact organism and the multitude of influences on endothelial cells *in vivo* (reviewed in Auerbach *et al*., 2003). Conversely, most *in vivo* systems do not permit the detailed visualization and functional dissection of cellular processes under defined conditions that allow reproducibility and interpretability of results. Mammals with cardiovascular mutations resulting in severe malfunction die very early in development, making functional analysis difficult; the generation of knockout mice and other mammalian models can be time consuming and costly, and the ability to perform molecular epistasis experiments and detailed analysis of effects on vessel morphology and cell identity is limited. The zebrafish has recently emerged as an experimentally and genetically accessible alternative model for studying vertebrate vascular formation both developmentally and in adult tissues. In this review, we highlight the advantageous features of the zebrafish and survey the experimental and genetic tools currently used to study blood vessel formation *in vivo*.

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Studying vascular development using the zebrafish

Zebrafish are small tropical freshwater fish capable of being housed in large numbers and in a relatively small space. They require approximately 3 months to reach sexual maturity, at which time females are capable of laying hundreds of eggs every few weeks. External fertilization permits immediate accessibility for experimentation and observation. Subsequent to fertilization, embryo development is rapid, and the embryos remain small enough to receive sufficient oxygen to develop for 3 – 4 days in the absence of a functional vascular network. This permits the identification and functional characterization of genes that significantly impair cardiovascular development. In addition, embryos and larvae remain optically clear, in large part due to the yolk sac existing separate from the body of the embryo. This transparency is perhaps the most significant feature of the zebrafish, as it permits real-time *in vivo* imaging at the cellular level *in vivo*.

The externally developing embryos of zebrafish are well suited for techniques such as microinjection of biologically active molecules like RNA, DNA, or interfering oligonucleotides (discussed in more detail later), microbead implantation, cell transplantation, fate mapping, and cell lineage tracing (Stainier *et al*., 1993; Holder & Xu, 1999; Mizuno *et al*., 1999; Reifers *et al*., 2000). These methodologies are also available in avian and amphibian model systems, although neither of these models provide both genetic accessibility and an optically clear embryo. These two qualities, combined with the ability of the zebrafish to survive severe cardiovascular defects for days (Stainier *et al*., 1996; Weinstein, 2002), have successfully facilitated unbiased forward-genetic screening for mutations affecting the circulatory system. Systematic random mutagenesis induced by chemical or insertional mutagens generates mutants that can then be identified through F3, haploid, gynogenetic diploid, or other screening methods (for a review, see Patton & Zon, 2001).

A variety of vascular-specific mutants have already been generated and studied in the zebrafish, including the *cloche*, *schwentine, gridlock, violet beauregarde, plcg(y10), kurzschluss*, and *out of bounds* mutants (Figure 1). *Cloche* mutants lack virtually all endothelial and circulating blood cells and are deficient in a novel gene functioning early in the 'hemangioblast' (Ribatti *et al*., 2002) progenitor of both lineages (Stainier *et al*., 1995; Liao *et al*., 1997; Thompson *et al*., 1998). *schwentine* mutants are defective in a zebrafish ortholog of *flk*1, the critical signaling receptor for the critical vascular factor vascular endothelial growth factor (VEGF)-A. An initial report showed that the *schwentine* gene product is essential for angiogenic processes, but not for angioblast specification in zebrafish (Habeck *et al*., 2002), suggesting differences in function compared to mammalian *flk1*. However, recent work has shown that there is a second zebrafish ortholog of *flk1* and morpholino knockdown of this gene in the *schwentine* mutant background leads to a more complete loss of angioblasts analogous to the phenotype of *Flk1*-knockout mice (Personal communication, Exelixis Inc.). The *gridlock, violet beauregarde, plcg(y10), kurzschluss*, and *out of bounds* mutants have no apparent defects in angioblast specification, but cause defects in the differentiation and/or patterning of blood vessels. *Gridlock (grl)* mutants lack trunk and tail circulation due to a failure to assemble the dorsal and lateral aortae properly (Weinstein *et al*., 1995), caused by a defect in a novel vascular bHLH factor related to mammalian HRT2 (Zhong *et al*., 2000). *Violet beauregarde (vbg)* mutants have circulatory defects associated with a massive enlargement of the central cranial vessels and improper arterial—venous (A – V) vascular connections (Roman & Weinstein, 2000). These mutants are defective in *alk1 (acvrl1)*, one of the several loci associated with a human autosomal-dominant congenital vascular disorder called 'hereditary hemorrhagic telangiectasia' (HHT, Johnson *et al*., 1996), making *vbg* mutant zebrafish a useful new genetic model for studying the genesis of this disorder. Individuals affected by HHT develop sporadically appearing and sometimes life-threatening enlarged A – V malformations (McDonald *et al*., 2000). *plcg(y10)* mutants are defective in zebrafish phospholipase C-γ1, an effector of receptor tyrosine kinase signaling, and are deficient in VEGF-mediated angiogenesis and arterial differentiation. The molecular nature of the defects in *kurzschluss (kus)* and *out of bounds (obd)* mutants has not yet been determined. In *kus* mutants, the posterior aortic arches (which normally go on to contribute to the branchial arteries of the gills) fail to form or remodel properly, and go on to develop A – V shunts, creating ineffective circulatory 'short cuts' that deplete circulation in the rest of the animal (Stainier *et al*., 1996). *obd* mutants also form the major primary vessels normally, but display premature sprouting and mispatterned growth of the trunk

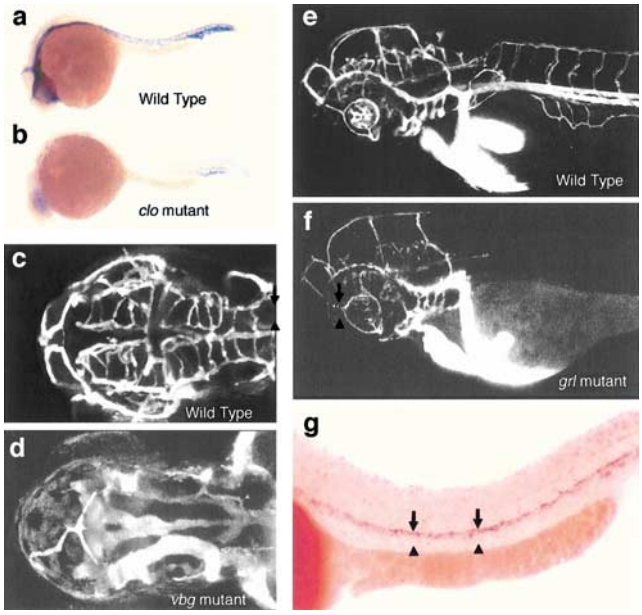

Figure 1 Vascular-specific zebrafish mutants identified by forward-genetic screening. A variety of different mutants affecting endothelial specification, differentiation, and patterning have been identified in the zebrafish, a few of which are shown here. *In situ* hybridization of 1-day-old wild-type (a) or *cloche (clo)* mutant (b) zebrafish with the vascular specific marker *flk1* shows that the blood vessels are virtually absent in mutants. Confocal microangiography of 2-day-old wild-type (c) and *violet beauregarde (vbg)* mutant (d) zebrafish reveals enlarged primary vessels and lack of perfused secondary vessels in mutants (images shown are dorsal views of the head). In contrast, confocal microangiography of wild-type (e) and *gridlock (grl)* mutant embryos (f) reveals relatively normal cranial vasculature, but lack of trunk and tail circulation due to defects in the morphogenesis of the dorsal aorta. The *grl* gene (g) is expressed in the trunk dorsal aorta (arrows), but not in the adjacent trunk cardinal vein (arrowheads). Anterior is to the left in all panels.

intersegmental vessels, secondary trunk vessels that normally appear regularly at each somite boundary (Childs *et al*., 2002).

In addition to being useful for forward-genetic screening for vascular-specific mutants, zebrafish are also amenable to generating transgenic animals. The application of transgenic technology to the zebrafish (Jowett, 1999) has resulted in the ability to create or enhance *in vivo* imaging capabilities and spatially and temporally control gene expression. Transgenic zebrafish lines expressing green fluorescent protein (GFP or enhanced (E)GFP) within vascular endothelial cells have been particularly useful for studying the formation of the vasculature *in vivo*. A murine *tie2* (a vascular-specific tyrosine kinase receptor activated by angiopoietin ligands) promoter construct successfully drives GFP expression in endothelial cells in mice and zebrafish, and stable germline transgenic lines have been prepared in both species (Motoike *et al*., 2000), although in zebrafish the murine promoter drives substantial nonvascular expression of GFP in the hindbrain and more posterior neural tube, and the overall level of expression is relatively low compared to that in mice. More recently, the zebrafish *fli1* (a transcription factor expressed in the presumptive hemangioblast lineage, and later restricted to vascular endothelium, cranial neural crest derivatives, and a small subset of myeloid derivatives) promoter, was used to generate a germline transgenic fish expressing EGFP (Figure 2) (Lawson & Weinstein, 2002b). These lines express EGFP at high levels in the vasculature, faithfully recapitulating the expression pattern of the endogenous *fli1* gene, and permit very high-resolution long-term time-lapse analysis *in vivo* of the endothelial cells in developing blood vessels.

Multiphoton confocal time-lapse imaging of Fli-EGFP transgenic zebrafish has enabled detailed analysis of both normal vascular development and defective vessel formation due to genetic or experimental perturbations. Lawson & Weinstein (2002b) showed *in vivo* that growing blood vessels are extremely active, extending and retracting filopodial processes up to tens of microns in all directions. Using Notch signaling-defective *mindbomb* (*mib*^{ta52b}) mutants on the Fli-EGFP background, they were able to characterize the specific vascular patterning defects associated with this mutant in the cranial vasculature and in the intersegmental vessels of the trunk vasculature. In a separate study (Isogai *et al*., 2003), Fli-EGFP transgenic zebrafish were used to examine the angiogenic vascular network assembly in the developing trunk, leading to a novel two-step model whereby a primary vascular network assembles from sprouts from the dorsal aorta followed by emergence of secondary vein-derived (posterior cardinal vein) sprouts that interact dynamically with the primary network to determine the final functional 'wiring' of the trunk network (Figure 2e). By crossing the Fli-EGFP fish to a mutant defective in cardiac troponin T called *silent heart (sih)* that lacks a heart beat and blood flow (Sehnert *et al*., 2002), these authors were able to assess the role of flow dynamics in the formation of the trunk vascular network. The results of these studies showed that while flow is not critical to primary and secondary sprouting and gross anatomical patterning of the trunk angiogenic vessels, it is probably a critical determinant of the interconnections between these vessels and of their final A – V identity.

Transgenic methods can also be used for functional manipulations in the zebrafish. The same promoters used to drive the expression of GFP and other fluorescent tracers (as described above) can also be used to drive the expression of functionally active genes in a tissue-specific manner. Transient, mosaic expression of genes can be achieved following microinjection of appropriate constructs into zebrafish. In cases where mosaic expression is undesirable, germline transgenic animals can be generated. Where the expression of functionally active molecules is not compatible with embryonic survival, methods such as binary transgenesis (in which separate 'activator' and 'effector' transgenic lines are generated and then intercrossed to express a target gene) such as the Gal4-UAS system can be employed for spatial and temporal control of gene expression. Binary transgenesis has been used to great effectiveness in mice (Lewandoski, 2001) and similar methods are now becoming available in the zebrafish (Scheer & Camnos-Ortega, 1999).

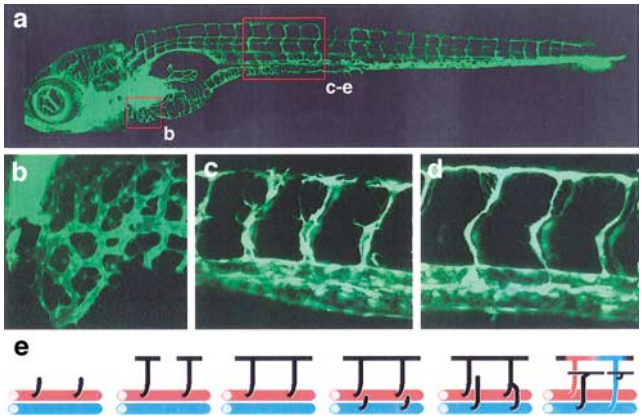

Figure 2 EGFP expression in the vasculature of live transgenic embryos and larvae permits dynamic imaging of developing blood vessels using multiphoton confocal microscopy. (a) The 7-day-old *TG(fli1:EGFP)*^{y1} zebrafish larva: boxed areas labeled (b) and (c – e) indicate approximate regions shown in the corresponding panels. (b) Higher magnification image of hepatic (liver) blood vessels in a 5-day-old transgenic larva. (c, d) Trunk vessels in approximately 1.25- and 1.5-day-old embryos, showing two stages in the formation of the dorsal-aorta-derived network of primary intersegmental vessels. Time-lapse imaging of trunk vessels such as these has been used to derive a 'two-step' model for trunk vascular network assembly shown in panel e (for a description of this model, see Isogai *et al*. (2003). Images in (a – d) are lateral views, anterior to the left. Panels a and b are from Lawson & Weinstein (2002b). Panels c – e are from Isogai *et al*. (2003).

The lessons learned in the zebrafish are likely to be readily transferred to other vertebrate organisms, including mammals, since the complex circulatory system of the zebrafish is in most respects quite similar to that of other vertebrates (Fishman, 2001; Isogai *et al*., 2001). A comparison between the blood vessels of zebrafish and other vertebrates presents a striking degree of anatomical and functional conservation of vascular pattern. For example, most of the cranial vessels present in the 2.5 – 3.5 dpf zebrafish are also found in other developing vertebrates. These vessels include the aortic arches, lateral dorsal aortae, internal carotid arteries, primordial hindbrain channel, anterior cardinal vein, and basilar artery. This conservation of vascular anatomy suggests that the vascular development is directed by genetically programmed, evolutionarily conserved control mechanisms, and that the information ascertained from the zebrafish is in accordance with existing information and should be readily transferable to other vertebrates. Indeed, it is clear from the analysis of a variety

of zebrafish orthologs of vascular-specific genes first described in other species that most of these genes have very similar spatial and temporal expression patterns in the fish (Roman & Weinstein, 2000). Some of the genetic control mechanisms responsible for regulating the expression of these genes and for vascular differentiation and patterning have now begun to be studied in the zebrafish.

Studying A – V fate determination using the zebrafish

The zebrafish has recently made important contributions to the identification of molecular pathways responsible for A – V differentiation of endothelial cells. In the past, the A – V fate of endothelial cells was believed to follow from physiological parameters such as differences in blood flow and pressure, but recent work has shown that early endothelial A – V differentiation is in fact genetically programmed. This early-programmed development of arterial and venous vessels is not precisely recapitulated in adult tissues, with shifts in blood flow and pressure and local environmental influences playing important deterministic roles. However, the molecular players that have been identified as playing an important role in A – V fate determination during early development are also expressed in adult arteries and veins and during pathologically associated vessel growth and have been shown to be functionally important in this context (Pola *et al*., 2001). It is therefore likely that successful therapeutic applications and establishing a vasculature during tissue regenerative processes or tissue engineering will require understanding these pathways and how to manipulate them both *in vivo* and *in vitro*.

Initial evidence that arterial and venous endothelial cells possess distinct molecular identities came from work with ephrin and Eph genes in mice (Wang *et al*., 1998b). Wang *et al*. (1998b) described the expression of ephrin B2 (*Efnb2*), a member of the ephrin family of membrane ligands. Prior to the onset of flow, *Efnb2* is expressed specifically in arterial endothelial cells and is absent in venous endothelial cells, while the ephrinB2 receptor Ephb4 is preferentially expressed in veins. Targeted gene deletion of each member of this ligand – receptor pair resulted in similar cardiovascular abnormalities, demonstrating their necessity and likely direct interaction, for normal vascular development (Wang *et al*., 1998b; Gerety & Anderson, 2002). Wang *et al*. (1998b) generated mutant mice with the *LacZ* gene 'knocked in' to the *ephrin B2* locus, and homozygous mutants continued to express *LacZ* appropriately in the arterial compartment, indicating that the actual molecular determination of arterial or venous fate involves additional factors upstream of *ephrin B2*.

Zebrafish studies have been critical in uncovering and dissecting the functional roles of these upstream factors, resulting in the identification of a signaling cascade for arterial fate determination consisting of sequential hedgehog, vascular endothelial growth factor, and notch signaling (Figure 3). A variety of studies in mammals and other vertebrates have revealed the specific expression of Notch signaling genes (Notch, Delta, Jagged, etc.) in arterial, but not in venous, endothelial cells, and murine knockout studies showed that these molecules play an important functional role in the vasculature (reviewed in Lawson & Weinstein, 2002a; Weinstein & Lawson, 2002). Although the nature of this functional role was not determined in the murine studies, their arterial-specific expression suggested that these genes might be playing a role in artery formation. A number of recent studies in the zebrafish (Zhong *et al*., 2000; Lawson *et al*., 2001; 2002) have now demonstrated that Notch signaling promotes arterial differentiation at the expense of venous differentiation during vascular development. As in other vertebrates, Notch signaling genes such as *notch5* (Kortschak *et al*., 2001) and *deltaC* (Smithers *et al*., 2000) are specifically expressed in arteries rather than veins in the zebrafish. To test its role in the vasculature, Notch signaling was repressed in zebrafish embryos either genetically, using the neurogenic *mindbomb (mib)* mutant, or experimentally, by injecting mRNA encoding a dominant-negative DNA-binding mutant of *Xenopus suppressor of hairless* protein (Lawson *et al*., 2001). In either case, the repression of Notch signaling resulted in loss of *ephrinB2a* expression from arteries and ectopic expansion of normally venous-restricted markers such as *ephb4* and *flt-4* into the arterial domain (Figure 3b – d). Conversely, the activation of Notch signaling suppressed the expression of vein-restricted markers and promoted ectopic expression of *ephrinB2a* and other arterial markers in venous vessels. This activation was accomplished either by heat-shock promoter-driven ubiquitous expression of the Notch1a intracellular domain (Notch1a-ICD) or by Fli1-promoter-driven vascular-specific expression of Notch5-ICD. The latter set of experiments demonstrated the vascular endothelial cell autonomy of

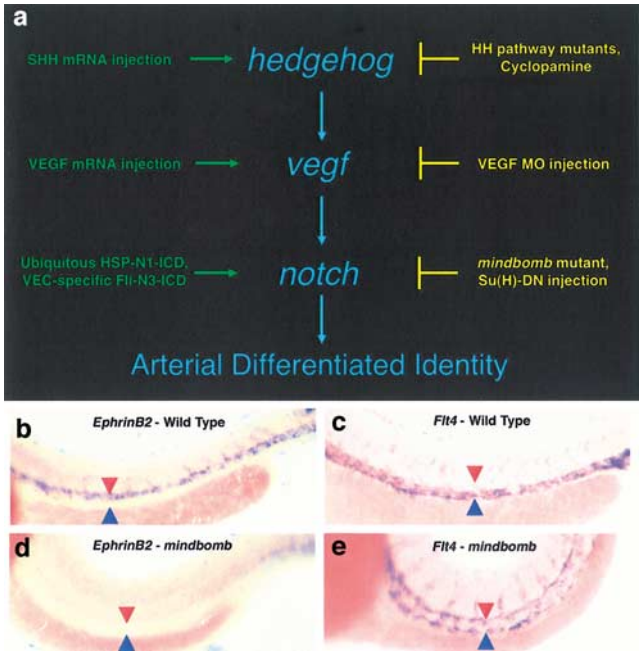

Figure 3 Zebrafish studies have led to the elucidation of a molecular pathway for arterial differentiation involving sequential hedgehog, VEGF, and Notch signaling (a; for details, see Lawson *et al*., 2001). An example of the data used to derive this model is shown in (b – e). In wild-type zebrafish the arterial marker *ephrinB2* is expressed in the dorsal aorta (red arrowheads), but not in the posterior cardinal vein (blue arrowheads) at 24 h postfertilization (b), while *flt4* is expressed in the vein, but not the aorta (c). In Notch signaling-deficient *mindbomb* mutant embryos, *ephrinB2* is no longer expressed in the aorta (d), while *flt4* expression is expanded to include both the posterior cardinal vein and the aorta (e). Panels b – e are modified from Lawson *et al*. (2001).

Notch-ICD effects, confirming that Notch is in fact acting at the level of the vascular endothelial cell itself and not *via* indirect signals from some other, adjacent Notch-responsive cells or tissues.

In addition to demonstrating that Notch signaling promotes arterial differentiation, Lawson *et al*. (2002) further dissected the A – V differentiation signaling hierarchy by demonstrating that *sonic hedgehog* (*shh*) and *vegf* act upstream of Notch. As in embryos lacking Notch signaling, embryos lacking *shh* or *vegf* fail to express *ephrin-B2a* within their blood vessels. The overexpression of shh promotes ectopic arterial vessel formation in the trunk, while the overexpression of vegf *via* injection of *vegf* mRNA suppresses the expression of vein-restricted markers and results in the expression of *ephrinB2a* and other arterial markers in venous vessels. Through 'molecular epistasis' experiments, Lawson *et al*. were able to determine that shh activity induces the expression of vegf in the somites, and that vegf then activates notch signaling in the endothelial cells of the developing dorsal aorta, promoting arterial differentiation. Recently, using genetic screening methods, Lawson and colleagues identified a zebrafish mutant deficient in both angiogenesis and arterial differentiation as a result of a defect in *phospholipase C gamma-1 (plcg1)* (Lawson *et al*., 2003). Phospholipase C genes are known effectors of signaling *via* receptor tyrosine kinases such as the vegf receptor Flk1, and the vascular expression of *plcg1* and vascular-specific phenotype of the mutant in this gene suggested that it might be functioning downstream of vegf signaling. Indeed, further experiments showed that *plcg1* mutants were insensitive to both angiogenic and arterial differentiation responses to vegf overexpression.

In support of the zebrafish findings regarding roles for hedgehog and vegf signaling in the vasculature, recent studies in mice have also implicated shh and vegf signaling in regulating blood vessel growth and arterial differentiation. Shh signaling has been shown in mice to influence vascular development, (Pepicelli *et al*., 1998; Rowitch *et al*., 1999) and a recent study evaluated the effect of shh in adult mouse tissues (Pola *et al*., 2001). Shh induces the expression of all three vegf-1 isoforms, angiopoietin 1, and angiopoietin 2 in ischemic limbs, and induced new blood vessel growth without affecting endothelial cell migration or proliferation (Pola *et al*., 2001). Mukouyama *et al*. (2002) evaluated the influence of the nervous system on blood vessel development, and found that peripheral nerves express vegf and influence vascular patterning and arteriogenesis in embryonic skin. Further, they demonstrated *in vitro* that vegf-expressing neurons and Schwann cells induced ephrinB2 expression in endothelial cells when cocultured, and that exogenously added vegf had a similar effect. They also demonstrated that a vegf-blocking antibody could abrogate this response. Two additional studies performed in adult animals suggest that vegf also plays a role in postnatal arterial differentiation. Visconti *et al*. (2002) evaluated the influence of overexpression of multiple classes of angiogenic factors. Of particular interest for this review, they showed that αMHC::VEGF transgenic mice expressing VEGF-A in the heart had an increased percentage of arterial (ephrin B2 +) vessels in the adult heart tissue compared to wild-type mice. In another study, Springer *et al*. (2003) demonstrated an increase in arterial concentration in adult skeletal muscle in response to VEGF-A expression. Transplantation of myoblasts expressing VEGF-A in nonischemic skeletal muscle resulted in an increased capillary density in the region of the implanted cells and a region of arteriogenic growth immediately adjacent to the implanted cells. The authors noted that this type of arteriogenic growth is distinct from that typically seen as a result of collateral arteriole formation because of its proximity to the site of vegf delivery in a region of tissue that has few, if any, pre-existing arteriolar vessels. This indicated that the arterial formation was a direct result of the presence of vegf.

Collectively, these studies demonstrate that the zebrafish is a useful model for uncovering novel signaling pathways regulating blood vessel formation and that the findings in zebrafish translate not only to other developing vertebrate models but also have relevance for adult vessel formation. This increases the likelihood that vascular findings in the zebrafish will have direct therapeutic implications. The specific advances in our understanding of the A – V differentiation pathway will be important in designing methods for directing organ-specific vessel growth for tissue regeneration, and for targeting specific vessel components for the inhibition of growth. It is anticipated that our knowledge and understanding in this area will only continue to grow with the zebrafish as an integral component, resulting in the identification of novel factors and the more subtle characterization of the various vessel types and their growth.

Zebrafish as a model for therapeutic angiogenesis applications

Although the zebrafish is rapidly becoming an established model for studying vascular development, its usefulness for applications relating more directly to therapeutic angiogenesis is less well established. As noted above, current therapeutic angiogenesis strategies are most commonly directed at either the inhibition of vessel growth in tumors, or the promotion of vessel growth in cases of either ischemic tissue repair/regeneration or tissue engineering, where synthetic or biological replacement tissues require a functional vasculature. Developing effective strategies requires a thorough understanding of pathological and physiological vessel growth and adaptation, and the zebrafish has provided important information regarding the fundamental aspects of vessel specification and differentiation, particularly with regard to A – V differentiation. However, the use of zebrafish in therapeutic angiogenesis applications can be extended beyond the identification of signaling mechanisms responsible for vascular development. The zebrafish has the potential to serve as an excellent model for high-throughput screening for substances with pro- or antiangiogenic activities, rapid evaluation of the efficacy and possible teratogenic side effects of identified substances, evaluation of cancer therapies, and study of thrombosis, to name just a few of the possible applications.

Zebrafish embryos and larvae are raised in an aqueous environment and are readily permeable to many different compounds in their culture media. By simply adding the chemicals or drugs to the zebrafish embryo culture water, potential pro- or antiangiogenic activities of these substances can be rapidly and easily evaluated along with the assessment of additional unwanted teratogenic or toxic side effects on other developing tissues. This method has already been successfully used to treat zebrafish with known inhibitors of

specific growth factors like vegf to study angiogenic signaling (Chan *et al*., 2002), or to study the effects of toxins on vascular development (Cheng *et al*., 2001). The availability of large 'chemical screening libraries' combined with the ease of screening for vascular and other defects in the small optically clear zebrafish embryo makes it possible to perform large-scale high-throughput chemical screens *in vivo* to identify new compounds with desired activities. A recent screen for chemicals causing specific developmental defects carried out by Peterson *et al*. (2001) identified a number of interesting novel chemicals with specific cardiovascular effects. Compound 31J6 caused aberrant 2 : 1 atrium to ventricle contraction ratios in the zebrafish heart, resembling a human cardiovascular condition called second-degree atrioventricular heart block. Embryos treated with compound 31J6 displayed cardiac fibrillation defects resembling those in zebrafish *breakdance* mutants. Another small molecule, dubbed concentramide, causes ventricles to form within the atrium (Peterson *et al*., 2001). These and other compounds were highly specific, eliciting their cardiovascular effects without significant effects on other tissues.

Another method for testing the effects of drugs and compounds on the vasculature that is not currently being applied to the zebrafish, but that could readily be adapted for use in this model, is the application of controlled release technology commonly involving the injection of loaded microspheres (Gupta & Ravi Kumar, 2000; Ravi Kumar, 2000). This method would have the advantage of allowing the evaluation of compounds that do not readily cross cell membranes. Microinjection techniques are routinely performed in zebrafish in order to treat embryos with biologically active molecules, mark cells for transplantation, and delineate the vasculature by injection of fluorescent microspheres for microangiography. Current protocols could be modified to include the injection of zebrafish embryos, larvae, or adults with loaded microspheres for evaluation of pro- or antiangiogenic factors, chemotactic effects, or tissue-specific effects of systemically harmful drugs. Although injection methods are of course not as simple and rapid as merely adding compounds to the culture media of developing embryos, they can readily be scaled up such that one person could inject more than 1000 or more embryos in a day.

Cardiovascular functional and adaptive evaluations in an animal as small as the zebrafish may seem like a daunting task; however, several microtechniques have been developed that make it possible to assess blood pressure, distribution, flow and velocity, blood cell concentration, cardiac output and volume, vascularization, vessel diameter and vessel density (reviewed in Schwerte & Fritsche, 2003). In addition to the vascular imaging methods involving transgenic fish described above, a digital motion analysis method that generates a cast of the vasculature by tracing the flow of erythrocytes (Schwerte & Pelster, 2000) is being used in several applications to assess vascular response and adaptation. Fritsche *et al*. (2000) used digital motion analysis to study the responsiveness of the zebrafish larvae vasculature to nitric oxide and epinephrine when neuronal regulation is scarce or absent. By microinjecting reagents directly into the dorsal artery and vein, they were able to demonstrate that both the arterial and venous vasculature are vasoresponsive to endogenously produced nitric oxide. This revealed that even at very early stages in vascular development, vascular tone is regulated through a complex interaction of vasoactive substances. Schwerte *et al*. (2003) used digital motion analysis to evaluate blood cell concentration and distribution following the incubation of fish in hypoxic conditions, and Pelster *et al*. (2003) used this method to evaluate shifts in tissue vessel density in response to exercise training. In other studies, cardiac morphology and associated blood pressures have been characterized in the zebrafish, enabling cardiac structure and function-related studies to be performed in living zebrafish embryos and larvae (Hu *et al*., 2000; 2001). The physiological measurement methods described above would be difficult to scale up to use for high-throughput screening, but they do provide the ability to measure the physiological function of the circulatory system, which in combination with the high-resolution vascular imaging methods available in the fish can be used to great effect.

Antiangiogenic therapies are primarily directed at the treatment of cancer, and recent work has begun to explore the use of the zebrafish as a model for cancer research. Although cancer progression in zebrafish is much less well understood, evidence shows that cancer is a naturally occurring genetic disease in fish as it is in humans (Walter & Kazianis, 2001) with histology that closely resembles that of human tumors (Spitsbergen *et al*., 2000). A 3-year retrospective study of abdominal tumors in zebrafish performed at the Marine Biological Laboratory in Woods Hole, MA, U.S.A. characterized six naturally occurring tumors in the zebrafish, including seminomas, dysgerminomas, and pancreatic tumors (Smolowitz *et al*., 2002). The objective of such a retrospective analysis is to identify specific strains and lineages of zebrafish displaying susceptibility to particular naturally occurring tumors for use as models for the study of cancer pathogenesis. In addition to naturally occurring tumors in zebrafish, chemically induced zebrafish cancers have also been described (Beckwith *et al*., 2000; Spitsbergen *et al*., 2000), and recently a stable transgenic T-cell leukemia-bearing zebrafish was reported (Langenau *et al*., 2003). While much of the interest in establishing the zebrafish as a model for tumor progression has been motivated by a desire to apply the genetic tools of the zebrafish to understanding tumor pathogenenesis, the fish also provides a potentially superb model for studying blood vessel progression within tumors and the effectiveness of antiangiogenic cancer therapies. The ability to visualize and monitor the growth of blood vessels easily in fli-EGFP transgenic zebrafish embryos and larvae zebrafish provides a particularly powerful tool for this purpose.

In addition, the vascular research being performed in the zebrafish with therapeutic implications is not solely angiogenesis related. Work in Pudur Jagadesswaran's laboratory at the University of Texas Health Sciences Center has established the zebrafish as a model for the study of hemostasis and thrombosis. In a series of publications, Jagadeeswaran has shown that the hemostatic pathway in fish is similar to that in humans (Jagadeeswaran *et al*., 1999; Sheehan *et al*., 2001), and has developed screening methods for defects in the extrinsic pathway of the coagulation cascade (Jagadeeswaran *et al*., 2000) as well as microassays to screen for thrombocyte function (Gregory & Jagadeeswaran, 2002). Having established the zebrafish thrombosis model, Jagadeeswaran is now taking advantage of the genetic accessibility of the zebrafish to identify novel genes involved in thrombosis (Gregory *et al*., 2002).

The techniques and applications described thus far have predominantly involved the embryonic or larval stage zebrafish (1 – 6 days postfertilization). While some of the techniques are in fact limited to these stages, many are not. This is important because the growth and behavior of developing vessels can differ in significant ways from that of vessels in adult tissues. Zebrafish do in fact provide a very useful and effective model for new vessel growth in adult tissues. Although zebrafish adults are not optically clear like zebrafish embryos and larvae, they do allow for effective visualization and experimental analysis of accessible blood vessels. The *TG(fli1:EGFP)^{y1}* transgenic zebrafish line described above maintains EGFP expression in the endothelium of adult blood vessels and can be used to visualize vessels in the scales, skin, fins, and other accessible tissues easily with very high resolution (Figure 4, Lawson & Weinstein, 2002). The simple cellular composition of the caudal fin (Becerra *et al*., 1983) and the ability of fish to regrow fins following amputation has already made this a useful tool for exploring the genetic and cellular mechanisms for tissue regeneration (Johnson & Weston, 1995; Johnson & Bennett, 1999). Fin regeneration is associated with and requires new vessel growth, but until recently blood vessel regeneration had not been described or experimentally studied in the regenerating fin model. *TG(fli1:EGFP)^{y1}* adult fish were recently used to visualize and describe the process of vessel morphogenesis during fin regeneration (C.-C. Huang & S. Johnson, unpublished results). During normal fin regeneration, the vasculature progresses through distinct and stereotypic stages of vessel growth and remodeling. Initial vascular regrowth (0 – 14 days postamputation) occurs with the formation and subsequent remodeling of a complex vascular plexus, although interestingly later (14+ days postamputation), growth occurs without plexus formation. The basis for this 'switch' in growth mechanisms remains to be explored. Vascular morphogenesis was also assessed in a temperature-sensitive fin regeneration mutant, *reg6* (Johnson & Weston, 1995), that forms blood blisters in fins challenged to regenerate at an elevated (restrictive) temperature. Vessels were examined in regenerating fins of *reg6* mutants by crossing this mutant into the *TG(fli1:EGFP)^{y1}* background. In mutants at restrictive temperature, the branching morphogenesis required for early plexus formation and vessel growth was impaired, although interestingly later vessel growth that occurs without plexus formation was not affected (Figure 4e,f). Although the findings of this study will require further investigation for their implications to become clear, this work is important in that it lays the groundwork for future studies using the zebrafish regenerating fin as a model for analysis of adult vessel growth.

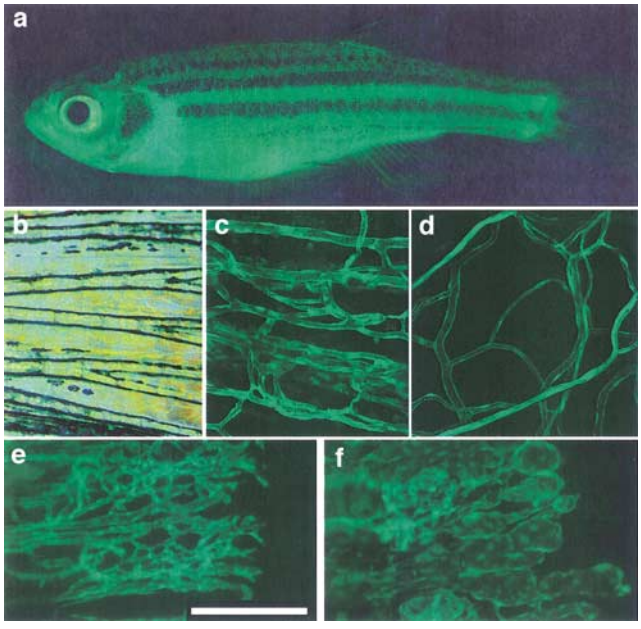

Figure 4 Imaging EGFP-positive blood vessels in living adult transgenic zebrafish. (a) Full-length view of a *TG(fli1:EGFP)^{y1}* adult fish reveals robust EGFP expression throughout the animal. Higher magnification images from the same fish (b – d) show that the EGFP expression is largely restricted to blood vessels. (b) Merged transmitted light and fluorescent image from the tail fin, showing vessels running along and between fin rays. (c) Confocal image of tail fin microvasculature, showing vascular-specific EGFP expression. (d) Confocal image of blood vessels associated with surface scales in the same adult fish. The *TG(fli1:EGFP)^{y1}* lines have proven useful in examining angiogenesis during the regeneration of severed tail fins in wild-type (e) or temperature-sensitive fin regeneration mutant (f) animals. Regenerating vessels in the mutant shown in panel (f) fail to branch properly and form enlarged vascular sinuses rather than properly patterned vascular plexuses as in wild-type animals. Panels a – d are from Lawson & Weinstein (2002b). Panels e and f are the courtesy of C.-C. Huang & S. Johnson.

Nonvascular-specific therapeutic applications of the zebrafish

Many of the methods and technologies discussed above are not limited to vascular applications, and the zebrafish has been promoted as a model for the development and/or testing of various nonvascular therapies as well. As noted above, the fish may serve as a valuable organism for the development of new and powerful cancer models. The existing cancer models in zebrafish provide a basis for the study of tumor pathogenesis at the molecular and organismal levels in the adult zebrafish. The ability to treat with chemical baths or microinject embryos makes the fish particularly well suited for application of high-throughput genetic and chemical screens (Peterson *et al*., 2000; Patton & Zon, 2001). Large-scale genetic and chemical screens can easily be established to identify new compounds that affect features of cancer such as excessive and rapid cell division and extensive angiogenesis (Amatruda *et al*., 2002). These sorts of screens could be performed to identify both the components that promote these phenotypes as well as those that reduce or eliminate them (by, for example, using a zebrafish line that is predisposed for tumor development in a hunt for suppressors). Adult zebrafish cancer models could also be developed in a similar manner to address the regulation of cell cycle control, multidrug resistance, chemoprevention, and antimetastasis (Eckhardt, 2002; Langheinrich *et al*., 2002; Yee *et al*., 2003).

The ongoing sequencing of the zebrafish genome and development of other genomic resources greatly strengthens the potential for zebrafish to continue to make significant contributions to the development of therapeutic applications. The sequence data already available have demonstrated that many genes are conserved between humans and zebrafish with large regions of their chromosomes showing synteny

(Fishman, 2001). This has resulted in characterization of mutant models that accurately represent particular human genetic disorders. For example, the zebrafish has provided the first genetically accurate animal model of hepatoerythropoietic porphyries, a congenital disorder resulting from deficiency in a particular enzyme in the ham biosynthetic pathway (Wang *et al*., 1998a). Zebrafish mutants corresponding to and/or modeling a variety of other human disorders have also been uncovered, including erythropoietic protoporphyria (Childs *et al*., 2000) and coarctation of the aorta (Weinstein *et al*., 1995) to name just a few (for a more complete review, see Barut & Zon, 2000; Shin & Fishman, 2002). Recently, microarray technology has expanded to the zebrafish with both cDNA and oligo arrays becoming available (Stickney *et al*., 2002; Ton *et al*., 2002; Lo *et al*., 2003). By combining contemporary molecular methods such as microarrays that make it possible to survey simultaneously a large number of genes at the same time with the powerful experimental and genetic tools available in the fish, it is to be expected that the fish will contribute significantly to our understanding of many different molecular pathways of interest to both basic scientists and clinicians.

Prospects

Therapeutic applications targeting the vasculature show promise in alleviating a wide variety of human pathologies, including strategies regulating vessel growth and differentiation as well as hemostasis and thrombosis. In the last several years, substantial progress has been made in our knowledge and understanding of the factors that regulate the vasculature. However, we still have only a limited understanding of how the actions of all of these different factors are coordinated *in vivo,* and this has in turn greatly limited our ability to develop viable therapeutic angiogenic applications. The zebrafish will continue to be a useful model for studying vascular development and deciphering the hierarchy of factors regulating vessel patterning and differentiation, but its usefulness for more direct development of angiogenic therapies remains relatively unexploited. Furthermore, many of the strengths of the zebrafish developmental model translate to the adult, expanding the scope of potential vascular research in the zebrafish. Together, these features make it likely that the popularity of the zebrafish as a tool for understanding and manipulating blood vessel formation will continue to expand.

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