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Zebrafish as a model system for the study of hemostasis and thrombosis

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Abstract

Purpose of review—Although the zebrafish has been established as a research tool over the past 2–3 decades, in hematology it has primarily been used to investigate areas distinct from coagulation. Advantages of this vertebrate model include high fecundity, rapid and external development, and conservation of virtually all clotting factors in the zebrafish genomic sequence. Here we summarize the growing application of this technology to the study of hemostasis and thrombosis.

Recent findings—Loss of function studies have demonstrated conservation of function for a number of zebrafish coagulation factors. These include positive and negative regulators of coagulation, as well as key components of the thrombus itself, such as von Willebrand factor, fibrinogen, and thrombocytes. Such analyses have also been leveraged to aid in the understanding of human variation and disease, as well as perform *in vivo* structure/function experiments.

Summary—The zebrafish is an organism that lends itself to a number of unique and powerful approaches not possible in mammals. This review demonstrates that there is a high degree of genetic and functional conservation of coagulation, portending future insights into hemostasis and thrombosis through use of this model.

Keywords

hemostasis; thrombosis; zebrafish; genetics

Introduction

The zebrafish (*Danio rerio*) is a small vertebrate tropical water fish that is now one of several favored systems for the study of human disease, with many unique advantages in comparison to mammalian models[1]. Zebrafish possess several characteristics that make it an ideal model to study in the laboratory. Adults are extremely fecund, with the ability to produce 200–300 offspring on a weekly basis. Embryonic development is external and transparent without any requirements for feeding. This accessibility facilitates both simple

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Conflicts of interest

There are no conflicts of interest.

and complex observations over the first week of life. During the embryonic/larval transition period (0–7 days post fertilization dpf), zebrafish are only millimeters (mm) in length, and hundreds of individuals can be easily maintained in 100 mm culture dishes. Rapid development of all major organ systems initiates during this period, easily observed under low power microscopy. Compared to mammals, a significantly greater number of adults can be bred and maintained in equivalent space.

Genomic conservation of hemostatic factors in zebrafish

Zebrafish share a high degree of genetic conservation with humans, including orthology to 70% of human genes[2*], although there are slight differences in nomenclature guidelines [3*]. The genome of the zebrafish contains many gene duplications, which has resulted in some neo functionalization and subfunctionalization[4]. The majority of coagulation factors appear to be single copy based on genomic sequence[5]. It has also been determined that the coagulation cascade is extensively conserved in another teleost fish, *Fugurubripes* (Fugu, puffer fish), with minimal coagulation factor duplication[6–8].

Genetic and small molecule screens for hematologic phenotypes in zebrafish

The use of zebrafish in hematology research was pioneered in the mid-1990s, primarily for analysis of hematopoiesis. Large-scale chemical mutagenesis screens employed a forward genetic approach, which led to the identification of many novel molecules regulating hematopoiesis[9,10]. This technology was later applied to other areas of hematology, and resulted in the discovery of a novel iron exporter, ferroportin[11], which was determined to be mutated in autosomal-dominant hemochromatosis[12]. The first genetic screen applied to hemostasis utilized laser-induced endothelial injury to induce occlusive thrombi in larvae. Reversal of this phenotype identified a mutant with linkage to the prothrombin(*f2*) locus[13]. Future screens are expected to result in the discovery of novel loci beyond the canonical coagulation cascade.

In addition to genetic screens, zebrafish are particularly suited to phenotype based, large-scale, small molecule screens in embryos and larvae, since these can be arrayed in multi well plates and readily absorb small molecules [14]. The translational potential of this approach has been demonstrated in hematopoiesis. A stable derivative of prostaglandin E₂(PGE₂) was identified as a hematopoietic stem cell regulator in zebrafish embryos, followed by confirmation using competitive transplantation in mice [15] and nonhuman primates [16]. This culminated in a successful phase I trial for *ex vivo* treatment of umbilical cord stem cells with PGE₂ prior to transplantation, which demonstrated clear safety as well as encouraging therapeutic potential[17**]. Given the data that show conservation of hemostatic pathways at both genetic and functional (see below) levels, there is similar potential to isolate novel regulators of hemostasis using zebrafish.

Analysis of coagulation factors in zebrafish demonstrates conservation of structure and function

Examination of specific zebrafish coagulation factors has demonstrated conservation with mammals at multiple levels. Until the relatively recent application of genome editing nucleases to gene targeting in zebrafish[18–21*], the vast majority of loss of function analyses were by knockdown using antisense morpholino oligonucleotides (MOs)[22]. Targeting of F2 using MOs demonstrated a bimodal phenotype [23] which bore a partial resemblance to the knockout of *F2* in mice [24,25]. Severe reduction resulted in morphological defects. These included retarded growth, brain and tail bud abnormalities at 1 dpf, followed by absence of circulating blood cells, reduced blood flow, pericardial edema, and truncal hemorrhage by 2 dpf. A subset of embryos did not exhibit morphologic defects, and intracranial hemorrhage was observed in 5–10% of this group. Acoagulopathic phenotype was detected through prolongation of the time to occlusion after laser-induced endothelial injury, and this was rescued by infusion of recombinant zebrafish F2.

Zebrafish factor VII (F7) was found to have a high degree of similarity with mammalian F7, and protein was detected in blood and liver [26]. As expected, F7 depleted plasma displayed a significant delay in fibrin generation. MO knockdown of F7 prolonged the laser injury induced time to occlusion in zebrafish larvae, consistent with a bleeding phenotype [27*]. Recent experiments have called into question whether the mammalian F7 activating protease (Fsap/habp2) truly activates F7 [28]. In support of these data, MO knockdown of Fsap in larvae did not affect either the ability to form a thrombus in response to endothelial injury or activation of F7 [27*]. In contrast, hepsin knockdown decreased F7 activation and inhibited induced thrombus formation [27*], results that are inconsistent with the mouse knockout [29].

Conservation of structure and function of the adhesive coagulation factors, von Willebrand factor (Vwf) and fibrinogen, has been demonstrated in zebrafish. Like humans, the zebrafish *vwf* locus consists of 52 exons, although it spans only 81 kilobases (kb) as opposed to 176 kb in humans [30,31]. Cloning of the cDNA demonstrated conservation of sequences required for propeptide and ADAMTS13 cleavage, and individual domain structures were conserved with 46% overall protein identity [31]. Expressed zebrafish *vwf* cDNA formed high molecular weight multimers and pseudo-Weibel-Palade bodies in cell culture[31]. Antisense knockdown mediated by MOs resulted in hemorrhage and loss of thrombocyte aggregation [32]. Taken together, these data demonstrate conservation of the key functions of VWF.

Fibrinogen is a hexameric protein formed as a homodimer of three polypeptide chains, and is encoded by the three loci, *FGA*, *FGB*, and *FGG*, which reside in a cluster on the long arm of human chromosome 4. The existence of three syntenic orthologs (*fga*, *fgb*, and *fgg*) to human fibrinogen was recognized through genomic sequencing[2] and confirmed by cytogenetic *in situ* hybridization [33]. The predicted amino acid sequences of zebrafish Fgb and Fgg share more than 50% identity with their human orthologs, while Fga is less well conserved [33]. In the case of the fibrinogen chains, mRNA *in situ* hybridization demonstrated conserved expression in liver, but also early expression in the yolk sac syncytial layer[33,34]. Expression of an Fgb-GFP (green fluorescent protein) fusion

demonstrated incorporation into a developing induced thrombus in larvae. MO knockdown of the three fibrinogen chains demonstrated intracranial and intramuscular hemorrhage, consistent with symptoms of human hypo- and a fibrinogenemia, while single deficiencies were less penetrant [34]. Although ablation of single chains in mice and humans completely eliminated fibrinogen production[35,36], MOs do not wholly eradicate target mRNA, a known shortcoming of this technology. Instead a complete knockout of *fga* was achieved using genome editing zinc finger nucleases (ZFNs), and resulted in overt hemorrhage in adult homozygous mutant fish [37*]. Variable lethality was observed in *fga*^{-/-} mutants, which is not surprising given the known heterozygosity of laboratory zebrafish [2,38]. This is consistent with the mouse *Fga* knockout, for which the genetic background altered survival [35].

The natural anticoagulant factors are conserved in zebrafish genomic sequence, including antithrombin III (*at3*)[39,40]. Targeted mutagenesis of *at3* using ZFNs resulted in adult lethality secondary to massive intracardiac thrombosis [40**]. While lethality was expected based on data from the mouse knockout [41] and clinical observations [42], this was in contrast to mammalian *in utero* lethality. Induction of thrombus formation by laser injury in 3 dpf larvae identified an unexpected prolongation of the time to occlusion, a bleeding phenotype. This was rescued by injection of human fibrinogen, consistent with a consumptive coagulopathy. Injection of fluorescently tagged human fibrinogen into larvae demonstrated disseminated intravascular coagulation with widespread fibrin clots. It was surprising that juvenile fish could tolerate what is a severe coagulation defect in mammals, suggesting the potential for species specific protective factors against this potent insult. The coagulopathy was also rescued by injection of plasmids expressing human *AT3*, and this was utilized as an *in vivo* platform to evaluate known *AT3* mutations. As expected, mutations in the P1 arginine abolished the ability to rescue, but surprisingly loss of the heparin binding site had no effect and was phenotypically normal. This demonstrates the value of this system for *in vivo* assessment of coagulation factor defects.

Thrombocytes: nucleated platelets

One specific area of interest has been the thrombocyte, with conservation of a number of platelet functions and regulatory processes. Unlike mammalian platelets, fish thrombocytes are nucleated [43]. Early work in trout demonstrated thrombocyte aggregation in response to thrombin and a thromboxane mimetic, U-46619, but not other eicosanoids [44]. The existence of an integrin-like fibrinogen receptor was revealed when it was shown that the tetrapeptide RGDS inhibited thrombocyte aggregation [45]. Ultrastructure analysis has verified that thrombocytes contain vesicles similar to the mammalian open canalicular system[46]. Despite the lack of a polyploid megakaryocyte-like stage in zebrafish, thrombopoietin (Tpo) and its receptor (Mpl) are conserved, and knockdown of the latter results in decreased circulating thrombocytes [47]. Zebrafish thrombocytes aggregate in response to platelet agonists (collagen, ADP, ristocetin, and arachidonic acid) and many receptors are conserved (ADP, collagen, VWF and thromboxane)[46]. Studies utilizing zebrafish thrombocyte have led to novel insights into platelet function and have been reviewed elsewhere[48,49].

Dissection of human coagulation and associated disorders using zebrafish

Coagulation is a multifaceted process, requiring contributions from cellular, vascular, and plasma protein elements. As demonstrated for At3, zebrafish present the opportunity to rapidly screen novel human sequence variants in such an *in vivo* context. This has been leveraged as an adjunct technique for positional cloning of factors affecting human platelets and their associated disorders. The first disease examined was human familial autosomal dominant thrombocytopenia. After localization of a locus on human chromosome 10p, the microtubule-associated serine/threonine-like kinase (MASTL) gene was identified as a candidate. Knockdown of the zebrafish ortholog with MOs resulted in reduction of circulating thrombocytes, as well as decreased expression of *itga2b* and *mpl*, providing supportive evidence [50]. MO knockdown in zebrafish also reinforced the discoveries of *NBEAL2* and *RBM8A* as the affected genes in the gray platelet and thrombocytopenia with absent radii syndromes, respectively [51,52].

Induced thrombosis in larval zebrafish has been used as a method to confirm targets identified through a systems biology approach. In one study, candidates were selected by virtue of megakaryocyte expression as compared to other hematopoietic lineages, sorted for those with transmembrane domains, with endothelial expression as a final selective criterion[53]. These putative novel platelet membrane proteins underwent functional screening *in vivo* by induction of arterial thrombosis following MO knockdown. Four novel genes (two that promoted thrombus formation, *BAMBI* and *LRRC32*, and two inhibitory, *ESAM* and *DCBLD2*) were discovered[53]. A similar approach used genome-wide platelet mRNA expression profiling in conjunction with association studies to detect potential targets. This was followed by induced thrombosis in zebrafish larvae, and identified *COMMD7* and *LRRFIP1* as potential enhancers of thrombus formation[54]. Gene silencing in zebrafish has also validated hits localized through meta-analyses of genome-wide association studies for platelet count and mean platelet volume[55]. MO knockdown in larvae confirmed 5 genes that regulated thrombopoiesis and/or erythropoiesis.

Conclusion

The zebrafish is a well-established vertebrate model organism with a number of unique and powerful advantages that make it a useful emerging tool for studying hemostasis. Studies to date have conclusively demonstrated significant homology with mammals. Functional conservation has been established for F2, F7, Vwf, fibrinogen, and Mpl, although there have been some unexpected differences in other factors. Knockdown of hepsin indicated a role in the initiation of coagulation by activation of F7, in contrast to studies in mice. At3 demonstrated that overall the effects of loss of function were conserved with mammals, but the temporal difference in phenotypic expression suggests that there is the potential to uncover novel biology.

The hemostatic system in zebrafish provides an opportunity to perform moderate to high throughput experiments in an intact organism, an advantage over *in vitro* or *ex vivo* systems. Traditional aspects of zebrafish investigation include genetic screens for modifier genes and evaluation of small molecules for novel therapeutics[1]. These have been highly successful

for other disciplines, including hematopoiesis, cardiology, and oncology [1], although it remains to be seen if this will be successful in hemostasis. In combination with standard knockdown and emerging genome editing approaches, zebrafish are exquisitely poised to filter systems biology pipelines, confirm candidate disease genes in positional cloning, and perform *in vivo* structure/function analyses for verification of human mutations or predictions from *in vitro* studies. These will enable further dissection of hemostasis, thrombosis, and platelet disorders with a throughput not available in larger animal models.

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Key points

- Zebrafish coagulation factors demonstrate structural and functional homology with their mammalian orthologs
- The initiation and endpoints of coagulation are conserved in zebrafish.
- Zebrafish present a unique and powerful system for the identification of novel therapeutics for hematologic disorders.
- Genome editing technology allows for rapid *in vivo* validation of coagulation factor structure/function in zebrafish.
- Putative human disease-causing variants can be rapidly confirmed in zebrafish