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Modeling Disorders of Blood Coagulation in the Zebrafish

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Abstract

Hemostasis, the process of blood clot formation and resolution in response to vascular injury, and thrombosis, the dysregulation of hemostasis leading to pathological clot formation, are widely studied. However, the genetic variability in hemostatic and thrombotic disorders is incompletely understood, suggesting that novel mediators have yet to be uncovered. The zebrafish is developing into a powerful in vivo model to study hemostasis, and its features as a model organism are well suited to (a) develop high-throughput screens to identify novel mediators of hemostasis and thrombosis, (b) validate candidate genes identified in human populations, and (c) characterize the structure/function relationship of gene products. In this review, we discuss conservation of the zebrafish hemostatic system, highlight areas for future study, and outline the utility of this model to study blood coagulation and its dysregulation.

Keywords

Zebrafish (*Danio rerio*); Blood coagulation; Thrombocytes; Hemostasis; High-throughput screening

Introduction

Since the first description of the coagulation cascade over a half century ago [1, 2], clinicians have developed the ability to measure various clotting proteins and establish diagnoses in affected patients suffering bleeding or thrombotic diatheses [3]. However, despite these useful tools, genetic variability often complicates the ability to interpret plasma coagulation factor levels [3, 4]. Affected patients with similar clotting ex vivo testing profiles may have significantly different clinical manifestations, with high degrees of variable expressivity and incomplete penetrance [3]. Mouse models have been used with

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Compliance with Ethics Guidelines

Human and Animal Rights and Informed Consent

This article does not contain any studies with human subjects performed by any of the authors. With regard to the authors' research cited in this paper, all institutional and national guidelines for the care and use of laboratory animals were followed.

Conflict of Interest

Colin A. Kretz declares no conflict of interest. Jordan A. Shavit has served as a consultant to CSL Behring and Octapharma regarding therapeutic products for patients with bleeding disorders. Angela C. Weyand declares no conflict of interest.

some success to identify genetic modifiers that may underlie this variability, but this approach is time consuming and costly [5–12].

The zebrafish (*Danio rerio*) is a small tropical freshwater fish of the minnow family that has gained popularity as a vertebrate animal model to study human disease [13]. Many of the features that make zebrafish ideal to study in the laboratory are related to husbandry as well as physical and developmental characteristics. First, adults can produce up to 200–300 offspring weekly, which make high-throughput genetic studies in zebrafish particularly robust. Second, embryonic development is external and transparent, allowing detailed observations with as little as a light microscope. Third, zebrafish embryos and larvae during this developmental period [0–7 days post-fertilization (dpf)] are millimeters (mm) in length, and a single 100 mm culture dish can support hundreds of individuals. Compared to other commonly used mammalian animal models, five to tenfold more adults can be housed in equivalent space and at lower costs. These advantages have facilitated massively high-throughput genetic screens for developmental phenotypes in zebrafish [14•]. This has translated into a significant expansion of our knowledge of basic human biology and led to important insights into the pathogenesis and treatment of human diseases. These advantages coupled with emerging genome editing technologies ensure that zebrafish will remain an important model organism. Recently, these tools have facilitated investigation into the processes of hemostasis and the pathogenesis of thrombotic disorders. This field has historically been underrepresented in use of the zebrafish model but is well positioned to take advantage of its unique and powerful capabilities.

Coagulation Factors are Highly Conserved in Zebrafish Genomic Sequence

The zebrafish genome is highly conserved with humans, and 70 % of human genes possess apparent zebrafish orthologs [15•]. Zebrafish experienced a massive genome duplication during evolution with the result that 30–40 % of genes remain duplicated [16•]. As a consequence, some genes have divided their functions between paralogs or acquired new roles. Although the majority of coagulation factors are present as a single copy, a few appear to be duplicated [17]. The blood coagulation system has also been shown to be conserved in other non-mammalian vertebrates, such as the teleost *Fugu rubripes* (Fugu, puffer fish) [18–20]. These data provided an initial foundation suggesting that the zebrafish model would be well suited for blood coagulation research.

Conservation of the Coagulation Cascade in Zebrafish

Zebrafish coagulation factor genes are highly conserved with their corresponding orthologs in humans. To study the impact of targeted gene knockdown, antisense morpholino oligonucleotides (MOs) [21] had been the technology of choice in the preceding decade. Reduction of prothrombin (F2) using MOs demonstrated a dual phenotype [22] with similarity to the mouse *F2* knockout [23, 24]. At 1 dpf, many embryos demonstrated severe growth retardation. This was followed on 2 dpf by bleeding along the trunk, with secondary anemia, compromised circulation, and pericardial edema. Of the embryos that did not exhibit these phenotypes, 5–10 % developed intracerebral hemorrhage and showed a lack of thrombus formation following laser-induced endothelial injury.

Zebrafish factor VII (F7) shares a high degree sequence homology to mammalian factor VII and was also shown to be produced and secreted into the circulation by the liver [25], akin to mammalian factor VII. Immunodepletion of F7 resulted in prolonged fibrin generation upon stimulation using thromboplastin as a source of tissue factor, demonstrating an intact and functionally conserved extrinsic coagulation pathway in zebrafish. Specific MO knockdown of *f7* prolonged the time to occlusion following laser injury in larvae, implying that a zebrafish ortholog to tissue factor can support F7-dependent coagulation in vivo [26]. Although an ortholog to F7-activating protease (Fsap/Habp2) is present in zebrafish, MO knockdown of *fsap* did not affect time to occlusion or activation of F7 [26]. Recent in vitro studies have also called into question the role for FSAP-mediated activation of F7 [27], in support of the findings in zebrafish. However, MO knockdown of the transmembrane serine protease *hepsin* resulted in decreased levels of activated F7, as well as prolongation of the laser-induced time to occlusion [26]. These results are inconsistent with data from the targeted *Hepsin* knockout mouse, which had no abnormalities in various clotting assays [28]. These conflicting data suggest that, despite sequence conservation with human orthologs, Hepsin and Fsap function might not be completely conserved between zebrafish and mammals. Alternative roles for these proteases in zebrafish might be relevant to human biology and warrant continued study.

von Willebrand factor (Vwf) is a critical protein responsible both for adhesion of platelets to the injured vessel wall and as a circulating chaperone for coagulation factor VIII [3, 29]. The human gene is extremely large and consists of 52 exons, with an excessively large exon 28. These features are duplicated in zebrafish, although the overall locus is compressed from 176 to 81 kilobases (kb) [30, 31]. Human VWF has a propeptide and is a target for cleavage by the protease ADAMTS13, features which appear to be conserved based on the *vwf* cDNA sequence [31]. Although the overall protein identity is 46 %, surprisingly the A1 and A2 domains (platelet binding and ADAMTS13 proteolysis, respectively) were the least conserved [31]. RNA in situ hybridization in embryos and larvae showed no signal in endothelial cells [31], although immunostaining with an anti-human VWF antibody detected widespread vascular expression [32]. Depletion of *vwf* by MO knockdown resulted in intracranial and yolk hemorrhage as well as impaired thrombocyte aggregation [32]. Expression of the zebrafish *vwf* cDNA in mammalian cell culture demonstrated assembly of multimers and pseudo-Weibel-Palade body-like organelles [31], both hallmarks of mammalian VWF. These data demonstrate conservation of the most essential roles of zebrafish and human VWF. This suggests that zebrafish may be a useful in vivo model for studying mutations within VWF that cause von Willebrand disease, the most commonly inherited bleeding disorder.

Fibrinogen is a hexamer that is assembled as a homodimer of three polypeptide chains, fibrinogen α, β, and γ (FGA, FGB, and FGG) [3, 33]. Genomic sequencing identified syntenic orthologs on zebrafish chromosome 1 (*fga*, *fgb*, and *fgg*) [15•]. This was also corroborated through cytogenetic in situ hybridization [34], although further study identified some differences. For example, the zebrafish *fgg* locus was noted to be spaced relatively farther from *fga* and *fgb* with two intervening genes, whereas the 3 loci are serially clustered in humans [34]. At the amino acid level, Fgb and Fgg are greater than 50 % identical to their

human orthologs, whereas Fga shares only ~34 % identity [34]. As expected, expression of all three chains was detected in hepatic cells through in situ hybridization of larvae, although there was signal in the syncytial layer surrounding the yolk [34, 35], consistent with changes in sites of gene expression during embryonic development. Liver-specific expression of an *fgb-egfp* (enhanced green fluorescent protein) transgene revealed functional conservation, as the expressed fusion protein accumulated in thrombi induced by laser-mediated endothelial injury [35]. Of note, adult transgenics showed occasional signs of external hemorrhage, suggesting functional dysfibrinogenemia due to the presence of the EGFP tag [35], serving as an important caution for modifications to the COOH-terminus of the fibrinogen molecule. Intraventricular and intramuscular hemorrhage were the primary phenotypes observed after individual and combined MO knockdown of the fibrinogen chains, strikingly similar to the symptomatology of patients with hypo- and afibrinogenemia [35].

The discovery and application of genome editing using zinc finger nucleases (ZFNs), TAL effector nucleases (TALENs), and CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats) RNA-guided nucleases to alter target sequences has great potential for rapid and robust gene disruption in zebrafish [36•, 37, 38, 39••]. These offer an alternative to MO technology, which can be unpredictable and often yields incomplete gene silencing. Recently, a systematic validation study of vascular phenotypes derived from MO knockdown in comparison to targeted genetic mutants derived through genome editing showed poor phenotypic correlation [40••]. Although the authors did not test for the potential off target effects of genome editing as an explanation for the discrepancies, the observed biological changes following MO-induced gene silencing are nonetheless called into question, at least for studies of vessel development.

A knockout of *fga* using ZFNs has been described [41•], and complete ablation of fibrinogen was demonstrated using a panel of anti-zebrafish fibrinogen antibodies. Overt hemorrhage was observed in adult homozygous mutant fish but not in embryos and larvae suggesting variable roles for fibrinogen during development. The reduced viability observed in the $fga^{-/-}$ mutant fish population was incompletely penetrant, which may reflect the known genomic heterozygosity of laboratory zebrafish [15•, 42]. In support of this conclusion, *Fga* ablation is variably lethal on different mouse genetic backgrounds [43].

In addition to known procoagulant effectors of the blood clotting system, zebrafish also express conserved natural anticoagulant factors, including antithrombin III (At3) [44, 45••]. Targeted ablation of *at3* using ZFNs was followed by spontaneous intracardiac thrombi with a variable range of lethality in adulthood [45••]. Although the endpoint of pathologic thrombus formation was consistent with the mouse knockout [46] and patient data [3], it occurred at a much later time point than the in utero lethality observed in mammals. Stimulation by laser-induced vascular injury in 3 dpf larvae resulted in the failure to form an occlusive thrombus, a phenotype more consistent with bleeding rather than thrombophilia. Infusion of plasma-derived human fibrinogen restored the ability to develop induced thrombi, suggestive of a consumptive coagulopathy in $at3^{-/-}$ mutant fish. Injection of fluorescently labeled human fibrinogen into larvae resulted in widespread fluorescence accumulation in the circulatory system of unprovoked $at3^{-/-}$ mutants. This would be expected in the syndrome of disseminated intravascular coagulation (DIC), a disorder

resulting from loss of regulation of the coagulation system [47]. Importantly, larval DIC was reversed by expression of human *AT3*, which was leveraged as an in vivo platform to study the impact of previously described *AT3* mutations [45••]. Mutations in the AT3 P1 arginine eliminated the capacity to rescue this phenotype, whereas disruption of the AT3 heparin binding site had no effect. These data highlight the power of coupling zebrafish and genome editing tools to study human diseases. Whole genome sequencing of human disease populations regularly uncovers genome sequence variants of unknown biological importance. As this technology is increasingly applied to the clinic, there is a recognized need for rapid and robust tools for evaluating the impact of gene sequence variants on protein function. Zebrafish may offer the unique capacity to rapidly characterize sequence variants in an in vivo setting.

Defining the Role of Thrombocytes in Hemostasis and Thrombosis

The closest equivalent to the platelet in zebrafish is the thrombocyte, a nucleated cell also found in birds [48], and evidence continues to support a functional correlation to mammalian platelets. Previous studies in trout (*Oncorhynchus mykiss*) established that like platelets thrombocytes respond to either thrombin or a thromboxane mimetic (U-46619), by forming aggregates [49]. Furthermore, other canonical platelet agonists (including collagen, ADP, ristocetin, and arachidonic acid) cause zebrafish thrombocytes to aggregate, and many receptors have been found to be conserved ($P2Y_{12}$, Gp6, Gp1b, and Tbxa2r, respectively) [50]. Thrombocyte aggregation can be inhibited by an RGDS tetra-peptide, suggesting a role for an integrin-like fibrinogen receptor during thrombocyte accumulation at sites of injury [51]. Although ultrastructure analysis has identified vesicles within thrombocytes that resemble platelet granules [50], more direct evidence for a fully functional open canalicular system is still needed.

Zebrafish lack an analog to the polyploid megakaryocyte, and thrombocyte production remains incompletely understood. Thrombopoietin (Tpo) and its receptor (Mpl) are conserved and MO knockdown of the latter decreased mobilization of a thrombocyte/erythroid precursor cell population (TEPs) from the extravascular compartment between the dorsal aorta and caudal vein [52]. Recently, TEPs were identified and isolated from zebrafish, and stimulation by zebrafish Tpo promoted thrombocyte differentiation, whereas stimulation by erythropoietin promoted erythroid differentiation [53]. The high-throughput screening capacity of zebrafish could provide a unique opportunity to identify novel mediators of thrombocyte development. Study of zebrafish thrombocytes has led to novel insights into platelet function (reviewed elsewhere [54, 55]), suggesting that results from thrombocyte production screens in zebrafish may be relevant to human biology.

Zebrafish Screens from Hematology to Hemostasis

The use of zebrafish as a model to study hematopoiesis is well established and serves as a proof-of-principle for guiding future discoveries in hemostasis. Forward genetic screens employing large-scale mutagenesis led to the identification of novel genes that regulate blood development [56, 57]. Furthermore, this approach has identified key mediators of human diseases, such as the link between ferroportin and hemochromatosis [58, 59]. The

first genetic screen in blood clotting employed laser-induced endothelial injury to produce thrombi in larvae, uncovering a mutation linked to the prothrombin (*f2*) locus [60]. These studies are facilitated by the highly fecund nature of zebrafish and the accessibility of their circulatory system to observation, features that are critical to hemostasis research.

Zebrafish also have been utilized for small molecule screens in both embryos and larvae [61]. For example, a stable derivative of prostaglandin E_2 (PGE_2) was shown to enhance hematopoietic stem cell engraftment in zebrafish embryos and mice [62]. This discovery was extended into nonhuman primates [63], followed by a small human clinical trial with umbilical cord blood transplantation [64•]. This example proves the feasibility of translating chemical screens performed in zebrafish into treatments for human diseases.

In hemostasis, recent experimentation testing the safety of cationic PAMAM dendrimers, an emerging basis for nanoparticles in biomedical applications, revealed substantial thrombocyte and fibrinogen aggregation akin to disseminated intravascular coagulation (DIC) [65]. This important toxicity result detected in zebrafish urges caution in moving forward to human trials for applications using this nanoparticle. With the conservation of the major components of the blood coagulation pathway, zebrafish are particularly well suited for screening small molecules that may evolve into the next generation of anticoagulants. Such a screen can readily be optimized to focus on targeting thrombosis-related pathologies while limiting the complication of bleeding that plagues the majority of traditional and new oral anticoagulants [66, 67].

Dissection of Human Coagulation and Associated Disorders Using Zebrafish

Blood coagulation requires well-timed orchestration of soluble plasma proteins with hematopoietic and vessel wall cellular mediators. As demonstrated for At3, zebrafish are uniquely poised to rapidly screen novel human sequence variants in an in vivo setting where the complexity of the entire circulatory systems comes to bear. This feature may be particularly useful in human genetics where validation of a genomic sequence variant linked to a particular disease is a major bottleneck. Zebrafish have assisted this process in a number of fields, including thrombocytopenia and associated disorders, as biochemical or cellular approaches are not always sufficient for such complex systems. Human familial autosomal-dominant thrombocytopenia was linked to a locus on chromosome 10p, and a missense mutation in microtubule-associated serine/threonine-like kinase (*MASTL*) was identified as a potential candidate gene. MO knockdown of the zebrafish ortholog of *MASTL* recapitulated clinical features, including thrombocyte deficiency and reduction of *itga2b* and *mpl* expression, consistent with a MASTL-dependent thrombocytopenia [68]. Similar experiments in zebrafish larvae also confirmed *NBEAL2* and *RBM8A* as the mutated loci in the gray platelet and thrombocytopenia with absent radii syndromes, respectively [69, 70].

Thrombosis models have been widely used in mice to study blood coagulation in an in vivo setting; however, these techniques are technically cumbersome and low throughput [71]. Induced thrombosis in zebrafish larvae offers many advantages and has been used to validate targets identified via systems biology. Targeted screening in zebrafish of five

predicted thrombocyte-expressed genes uncovered a previously unappreciated role for BAMBI (bone morphogenic protein and activin membrane-bound inhibitor) in laser-induced thrombus formation [72]. Subsequent studies in mice validated these results and determined that BAMBI expressed on the vessel wall, and not platelets, supports thrombus stability following laser-induced injury [73]. Zebrafish have also been used to validate potential mediators of thrombus formation identified from platelet mRNA expression profiling or genome-wide association studies (GWAS). These studies identified *COMMD7* and *LRRFIP1* as genes that contribute to thrombus formation [74]. Furthermore, zebrafish were successfully used to validate hits identified via GWAS meta-analyses for platelet count and mean platelet volume [75], providing a highly tractable system for validation of genomic signals from human populations.

Conclusion

Over the last two decades, the zebrafish has been established as a vertebrate organism with significant benefits beyond traditional mammalian models. The work summarized above demonstrates that it is a useful tool for the study of hemostasis. However, success in these endeavors will depend on continued delineation of the conservation among the key mediators of the blood clotting system. Such conservation provides the rationale for employing some of the most powerful high-throughput screening tools available to the zebrafish investigator. Zebrafish are particularly well suited for small molecule screens [13] that may identify novel anticoagulants. Forward genetic screening may uncover previously unknown mediators of thrombosis and thus possible new targets for future anticoagulant therapy. Furthermore, combined with emerging genome editing approaches, zebrafish are a convenient model to test hypotheses uncovered by systems biology pipelines, validate candidate disease genes, and study structure/function relationships for proteins in an in vivo setting. Leveraging the power of zebrafish offers the opportunity to enhance future research into the regulation of hemostasis and pathogenesis of thrombotic disorders that may ultimately improve the quality of diagnosis and treatment of important human diseases.

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References

Papers of particular interest, published recently, have been highlighted as:

• Of importance

•• Of major importance

1. Macfarlane RG. An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. Nature. 1964; 202:498–499. [PubMed: 14167839]
2. Davie EW, Ratnoff OD. Waterfall sequence for intrinsic blood clotting. Science. 1964; 145:1310–1312. [PubMed: 14173416]

3. Shavit, JA.; Ginsburg, D. Hemophilias and other disorders of hemostasis. In: Rimoin, DL.; Pyeritz, RE.; Korf, B., editors. Emery and Rimoin's principles and practice of medical genetics. 6. Elsevier Science; 2013. p. 1-33.

4. Ginsburg D. Identifying novel genetic determinants of hemostatic balance. J Thromb Haemost. 2005; 3:1561–1568. [PubMed: 16102020]

5. Lemmerhirt HL, Broman KW, Shavit JA, Ginsburg D. Genetic regulation of plasma von Willebrand factor levels: quantitative trait loci analysis in a mouse model. J Thromb Haemost. 2007; 5:329–335. [PubMed: 17155961]

6. Lemmerhirt HL, Shavit JA, Levy GG, Cole SM, Long JC, Ginsburg D. Enhanced VWF biosynthesis and elevated plasma VWF due to a natural variant in the murine Vwf gene. Blood. 2006; 108:3061–3067. [PubMed: 16873672]

7. Shavit JA, Manichaikul A, Lemmerhirt HL, Broman KW, Ginsburg D. Modifiers of von Willebrand factor identified by natural variation in inbred strains of mice. Blood. 2009; 114:5368–5374. [PubMed: 19789385]

8. Mohlke KL, Purkayastha AA, Westrick RJ, Smith PL, Petryniak B, Lowe JB, Ginsburg D. Mvwf, a dominant modifier of murine von Willebrand factor, results from altered lineage-specific expression of a glycosyltransferase. Cell. 1999; 96:111–120. [PubMed: 9989502]

9. Schlachterman A, Schuettrumpf J, Liu JH, Furlan Freguia C, Toso R, Poncz M, Camire RM, Arruda VR. Factor V Leiden improves in vivo hemostasis in murine hemophilia models. J Thromb Haemost. 2005; 3:2730–2737. [PubMed: 16359510]

10. Eitzman DT, Westrick RJ, Bi X, Manning SL, Wilkinson JE, Broze GJ, Ginsburg D. Lethal perinatal thrombosis in mice resulting from the interaction of tissue factor pathway inhibitor deficiency and factor V Leiden. Circulation. 2002; 105:2139–2142. [PubMed: 11994245]

11. Rosen ED, Xuei X, Suckow M, Edenberg H. Searching for hemostatic modifier genes affecting the phenotype of mice with very low levels of FVII. Blood Cells Mol Dis. 2006; 36:131–134. [PubMed: 16524747]

12. Cheli Y, Jensen D, Marchese P, Habart D, Wiltshire T, Cooke M, Fernandez JA, Ware J, Ruggeri ZM, Kunicki TJ. The modifier of hemostasis (Mh) locus on chromosome 4 controls in vivo hemostasis of Gp6−/− mice. Blood. 2008; 111:1266–1273. [PubMed: 17991808]

13. Santoriello C, Zon LI. Hooked! Modeling human disease in zebrafish. J Clin Invest. 2012; 122:2337–2343. [PubMed: 22751109]

14•. Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, Odenthal J, van Eeden FJ, Jiang YJ, Heisenberg CP, et al. The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. Development. 1996; 123:1–36. Often referred to as "The Screen", the entire edition of the journal Development was dedicated to a series of papers describing a large scale mutagenesis screen in zebrafish for developmental and morphological phenotypes. [PubMed: 9007226]

15•. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, Collins JE, Humphray S, McLaren K, Matthews L, et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 2013; 496:498–503. Publication of a high quality sequence assembly of the zebrafish genome. [PubMed: 23594743]

16•. Postlethwait JH. The zebrafish genome: a review and msx gene case study. Genome Dyn. 2006; 2:183–197. Describes a genome duplication event in zebrafish and posits neo- and sub-functionalization of the paralogs. [PubMed: 18753779]

17. Hanumanthaiah R, Day K, Jagadeeswaran P. Comprehensive analysis of blood coagulation pathways in teleostei: evolution of coagulation factor genes and identification of zebrafish factor VIIi. Blood Cells Mol Dis. 2002; 29:57–68. [PubMed: 12482404]

18. Davidson CJ, Hirt RP, Lal K, Snell P, Elgar G, Tuddenham EG, McVey JH. Molecular evolution of the vertebrate blood coagulation network. Thromb Haemost. 2003; 89:420–428. [PubMed: 12624623]

19. Davidson CJ, Tuddenham EG, McVey JH. 450 million years of hemostasis. J Thromb Haemost. 2003; 1:1487–1494. [PubMed: 12871284]

20. Jiang Y, Doolittle RF. The evolution of vertebrate blood coagulation as viewed from a comparison of puffer fish and sea squirt genomes. Proc Natl Acad Sci USA. 2003; 100:7527–7532. [PubMed: 12808152]
21. Bill BR, Petzold AM, Clark KJ, Schimmenti LA, Ekker SC. A primer for morpholino use in zebrafish. Zebrafish. 2009; 6:69–77. [PubMed: 19374550]
22. Day K, Krishnegowda N, Jagadeeswaran P. Knockdown of prothrombin in zebrafish. Blood Cells Mol Dis. 2004; 32:191–198. [PubMed: 14757435]
23. Sun WY, Witte DP, Degen JL, Colbert MC, Burkart MC, Holmback K, Xiao Q, Bugge TH, Degen SJ. Prothrombin deficiency results in embryonic and neonatal lethality in mice. Proc Natl Acad Sci USA. 1998; 95:7597–7602. [PubMed: 9636195]
24. Xue J, Wu Q, Westfield LA, Tuley EA, Lu D, Zhang Q, Shim K, Zheng X, Sadler JE. Incomplete embryonic lethality and fatal neonatal hemorrhage caused by prothrombin deficiency in mice. Proc Natl Acad Sci USA. 1998; 95:7603–7607. [PubMed: 9636196]
25. Sheehan J, Templer M, Gregory M, Hanumanthaiah R, Troyer D, Phan T, Thankavel B, Jagadeeswaran P. Demonstration of the extrinsic coagulation pathway in teleostei: identification of zebrafish coagulation factor VII. Proc Natl Acad Sci USA. 2001; 98:8768–8773. [PubMed: 11459993]
26. Khandekar G, Jagadeeswaran P. Role of hepsin in factor VII activation in zebrafish. Blood Cells Mol Dis. 2014; 52:76–81. [PubMed: 23954211]
27. Stavenuiter F, Dienava-Verdoold I, Boon-Spijker MG, Brinkman HJ, Meijer AB, Mertens K. Factor seven activating protease (FSAP): does it activate factor VII? J Thromb Haemost. 2012; 10:859–866. [PubMed: 22235940]
28. Wu Q, Yu D, Post J, Halks-Miller M, Sadler JE, Morser J. Generation and characterization of mice deficient in hepsin, a hepatic transmembrane serine protease. J Clin Invest. 1998; 101:321–326. [PubMed: 9435303]
29. Montgomery, RR.; Gill, JC.; Di Paola, J. Hemophilia and von Willebrand disease. In: Orkin, SH.; Nathan, DG.; Ginsburg, D.; Look, AT.; Fisher, DE.; Lux, SE., editors. Nathan and Oski's hematology of infancy and childhood. 7. Saunders Elsevier; Philadelphia: 2009. p. 1488-1525.
30. Dang LT, Purvis AR, Huang RH, Westfield LA, Sadler JE. Phylogenetic and functional analysis of histidine residues essential for pH-dependent multimerization of von Willebrand factor. J Biol Chem. 2011; 286:25763–25769. [PubMed: 21592973]
31. Ghosh A, Vo A, Twiss BK, Kretz CA, Jozwiak MA, Montgomery RR, Shavit JA. Characterization of zebrafish von Wille-brand factor reveals conservation of domain structure, multi-merization, and intracellular storage. Adv Hematol. 2012; 2012:214209. [PubMed: 23049555]
32. Carrillo M, Kim S, Rajpurohit SK, Kulkarni V, Jagadeeswaran P. Zebrafish von Willebrand factor. Blood Cells Mol Dis. 2010; 45:326–333. [PubMed: 21035359]
33. de Moerloose P, Neerman-Arbez M. Congenital fibrinogen disorders. Semin Thromb Hemost. 2009; 35:356–366. [PubMed: 19598064]
34. Fish RJ, Vorjohann S, Bena F, Fort A, Neerman-Arbez M. Developmental expression and organisation of fibrinogen genes in the zebrafish. Thromb Haemost. 2012; 107:158–166. [PubMed: 22116349]
35. Vo AH, Swaroop A, Liu Y, Norris ZG, Shavit JA. Loss of fibrinogen in zebrafish results in symptoms consistent with human hypofibrinogenemia. PLoS ONE. 2013; 8:e74682. [PubMed: 24098662]
36•. Meng X, Noyes MB, Zhu LJ, Lawson ND, Wolfe SA. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. Nat Biotechnol. 2008; 26:695–701. One of the first examples of targeted gene disruption in zebrafish using genome editing nucleases. [PubMed: 18500337]
37. Foley JE, Yeh JR, Maeder ML, Reyon D, Sander JD, Peterson RT, Joung JK. Rapid mutation of endogenous zebrafish genes using zinc finger nucleases made by Oligomerized Pool ENgineering (OPEN). PLoS ONE. 2009; 4:e4348. [PubMed: 19198653]
38. Sander JD, Cade L, Khayter C, Reyon D, Peterson RT, Joung JK, Yeh JR. Targeted gene disruption in somatic zebrafish cells using engineered TALENs. Nat Biotechnol. 2011; 29:697–698. [PubMed: 21822241]

39••. 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. Nat Biotechnol. 2013; 31:227–229. The first example of using CRISPR/Cas9 for targeted gene disruption in zebrafish. The relative simplicity of the technology suggested that this system would become widely available to zebrafish investigators and the method of choice to produce knockouts. [PubMed: 23360964]

40••. Kok FO, Shin M, Ni CW, Gupta A, Grosse AS, van Impel A, Kirchmaier BC, Peterson-Maduro J, Kourkoulis G, Male I, et al. Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. Dev Cell. 2015; 32:97–108. A detailed comparison of morpholino induced gene silencing and targeted knockout phenotypes in studies of the zebrafish vasculature. The results show poor correlation between the two technologies, suggesting that at least in this area of inquiry phenotypes observed from morpholino knockdown may require confirmation by genetic ablation. [PubMed: 25533206]

41•. Fish RJ, Di Sanza C, Neerman-Arbez M. Targeted mutation of zebrafish fga models human congenital afibrinogenemia. Blood. 2014; 123:2278–2281. The first example of a coagulation factor knockout using genome editing nucleases. [PubMed: 24553182]

42. Guryev V, Koudijs MJ, Berezikov E, Johnson SL, Plasterk RH, van Eeden FJ, Cuppen E. Genetic variation in the zebrafish. Genome Res. 2006; 16:491–497. [PubMed: 16533913]

43. Suh TT, Holmback K, Jensen NJ, Daugherty CC, Small K, Simon DI, Potter S, Degen JL. Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. Genes Dev. 1995; 9:2020–2033. [PubMed: 7649481]

44. Kumar A, Bhandari A, Sarde SJ, Goswami C. Sequence, phylogenetic and variant analyses of antithrombin III. Biochem Biophys Res Commun. 2013; 440:714–724. [PubMed: 24121110]

45••. Liu Y, Kretz CA, Maeder ML, Richter CE, Tsao P, Vo AH, Huarng MC, Rode T, Hu Z, Mehra R, et al. Targeted mutagenesis of zebrafish antithrombin III triggers disseminated intravascular coagulation and thrombosis, revealing insight into function. Blood. 2014; 124:142–150. Targeted gene disruption of at3 in zebrafish using zinc finger nucleases reveals an unexpected DIC phenotype. This paper also demonstrates the ease of assessing structure/function relationships of a coagulation factor using zebrafish larvae as an in vivo model system. [PubMed: 24782510]

46. Ishiguro K, Kojima T, Kadomatsu K, Nakayama Y, Takagi A, Suzuki M, Takeda N, Ito M, Yamamoto K, Matsushita T, et al. Complete antithrombin deficiency in mice results in embryonic lethality. J Clin Investig. 2000; 106:873–878. [PubMed: 11018075]

47. Liebman, HA.; Weitz, IC. Disseminated intravascular coagulation. In: Hoffman, R.; Benz, EJ.; Shattil, SJ.; Furie, B.; Cohen, HJ.; McGlave, P., editors. Hoffman hematology: basic principles and practice. 4. Churchill Livingstone; Philadelphia: 2005. p. 2169-2182.

48. Carradice D, Lieschke GJ. Zebrafish in hematology: sushi or science? Blood. 2008; 111:3331–3342. [PubMed: 18182572]

49. Lloyd-Evans P, Barrow SE, Hill DJ, Bowden LA, Rainger GE, Knight J, Rowley AF. Eicosanoid generation and effects on the aggregation of thrombocytes from the rainbow trout, *Oncorhynchus mykiss*. Biochim Biophys Acta. 1994; 1215:291–299. [PubMed: 7811714]

50. Jagadeeswaran P, Sheehan JP, Craig FE, Troyer D. Identification and characterization of zebrafish thrombocytes. Br J Haematol. 1999; 107:731–738. [PubMed: 10606877]

51. Hill DJ, Rowley AF. Are integrins involved in the aggregatory and phagocytic behaviour of fish haemostatic cells? J Exp Biol. 1998; 201:599–608. [PubMed: 9438834]

52. Lin HF, Traver D, Zhu H, Dooley K, Paw BH, Zon LI, Handin RI. Analysis of thrombocyte development in CD41-GFP transgenic zebrafish. Blood. 2005; 106:3803–3810. [PubMed: 16099879]

53. Svoboda O, Stachura DL, Machonova O, Pajer P, Brynda J, Zon LI, Traver D, Bartunek P. Dissection of vertebrate hematopoiesis using zebrafish thrombopoietin. Blood. 2014; 124:220–228. [PubMed: 24869937]

54. Lang MR, Gihr G, Gawaz MP, Muller II. Hemostasis in *Danio rerio*: is the zebrafish a useful model for platelet research? J Thromb Haemost. 2010; 8:1159–1169. [PubMed: 20180901]

55. Khandekar G, Kim S, Jagadeeswaran P. Zebrafish thrombocytes: functions and origins. Adv Hematol. 2012; 2012:857058. [PubMed: 22778746]

56. Weinstein BM, Schier AF, Abdelilah S, Malicki J, Solnica-Krezel L, Stemple DL, Stainier DY, Zwartkruis F, Driever W, Fishman MC. Hematopoietic mutations in the zebrafish. Development. 1996; 123:303–309. [PubMed: 9007250]
57. Ransom DG, Haffter P, Odenthal J, Brownlie A, Vogelsang E, Kelsh RN, Brand M, van Eeden FJ, Furutani-Seiki M, Granato M, et al. Characterization of zebrafish mutants with defects in embryonic hematopoiesis. Development. 1996; 123:311–319. [PubMed: 9007251]
58. Donovan A, Brownlie A, Zhou Y, Shepard J, Pratt SJ, Moynihan J, Paw BH, Drejer A, Barut B, Zapata A, et al. Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. Nature. 2000; 403:776–781. [PubMed: 10693807]
59. Montosi G, Donovan A, Totaro A, Garuti C, Pignatti E, Cassanelli S, Trenor CC, Gasparini P, Andrews NC, Pietrangelo A. Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (SLC11A3) gene. J Clin Invest. 2001; 108:619–623. [PubMed: 11518736]
60. Gregory M, Hanumanthaiah R, Jagadeeswaran P. Genetic analysis of hemostasis and thrombosis using vascular occlusion. Blood Cells Mol Dis. 2002; 29:286–295. [PubMed: 12547218]
61. Bowman TV, Zon LI. Swimming into the future of drug discovery: in vivo chemical screens in zebrafish. ACS Chem Biol. 2010; 5:159–161. [PubMed: 20166761]
62. North TE, Goessling W, Walkley CR, Lengerke C, Kopani KR, Lord AM, Weber GJ, Bowman TV, Jang IH, Grosser T, et al. Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. Nature. 2007; 447:1007–1011. [PubMed: 17581586]
63. Goessling W, Allen RS, Guan X, Jin P, Uchida N, Dovey M, Harris JM, Metzger ME, Bonifacino AC, Stroncek D, et al. Prostaglandin E2 enhances human cord blood stem cell xeno-transplants and shows long-term safety in preclinical nonhuman primate transplant models. Cell Stem Cell. 2011; 8:445–458. [PubMed: 21474107]
64•. Cutler C, Multani P, Robbins D, Kim HT, Le T, Hoggatt J, Pelus LM, Desponts C, Chen YB, Rezner B, et al. Prostaglandin-modulated umbilical cord blood hematopoietic stem cell transplantation. Blood. 2013; 122:3074–3081. A phase I clinical trial for PGE2-assisted allogenic hematopoietic stem cell implantation. The first successful demonstration of a novel therapeutic discovered in zebrafish being applied to humans. [PubMed: 23996087]
65. Jones CF, Campbell RA, Brooks AE, Assemi S, Tadjiki S, Thiagarajan G, Mulcock C, Weyrich AS, Brooks BD, Ghandehari H, et al. Cationic PAMAM dendrimers aggressively initiate blood clot formation. ACS Nano. 2012; 6:9900–9910. [PubMed: 23062017]
66. Weitz JI, Eikelboom JW, Samama MM. New antithrombotic drugs: antithrombotic therapy and prevention of thrombosis, 9th ed: American College of Chest Physicians evidence-based clinical practice guidelines. Chest. 2012; 141:e120S–e151S. [PubMed: 22315258]
67. Yeh CH, Gross PL, Weitz JI. Evolving use of new oral anticoagulants for treatment of venous thromboembolism. Blood. 2014; 124:1020–1028. [PubMed: 24923298]
68. Johnson HJ, Gandhi MJ, Shafizadeh E, Langer NB, Pierce EL, Paw BH, Gilligan DM, Drachman JG. In vivo inactivation of MASTL kinase results in thrombocytopenia. Exp Hematol. 2009; 37:901–908. [PubMed: 19460416]
69. Albers CA, Cvejic A, Favier R, Bouwmans EE, Alessi MC, Bertone P, Jordan G, Kettleborough RN, Kiddle G, Kostadima M, et al. Exome sequencing identifies NBEAL2 as the causative gene for gray platelet syndrome. Nat Genet. 2011; 43:735–737. [PubMed: 21765411]
70. Albers CA, Paul DS, Schulze H, Freson K, Stephens JC, Smethurst PA, Jolley JD, Cvejic A, Kostadima M, Bertone P, et al. Compound inheritance of a low-frequency regulatory SNP and a rare null mutation in exon-junction complex subunit RBM8A causes TAR syndrome. Nat Genet. 2012; 44(435–439):S431–S432.
71. Westrick RJ, Winn ME, Eitzman DT. Murine models of vascular thrombosis (Eitzman series). Arterioscler Thromb Vasc Biol. 2007; 27:2079–2093. [PubMed: 17600224]
72. O'Connor MN, Salles II, Cvejic A, Watkins NA, Walker A, Garner SF, Jones CI, Macaulay IC, Steward M, Zwaginga JJ, et al. Functional genomics in zebrafish permits rapid characterization of novel platelet membrane proteins. Blood. 2009; 113:4754–4762. [PubMed: 19109564]
73. Salles C II, Monkman JH, Ahnstrom J, Lane DA, Crawley JT. Vessel wall BAMBI contributes to hemostasis and thrombus stability. Blood. 2014; 123:2873–2881. [PubMed: 24627527]

74. Goodall AH, Burns P, Salles I, Macaulay IC, Jones CI, Ardissino D, de Bono B, Bray SL, Deckmyn H, Dudbridge F, et al. Transcription profiling in human platelets reveals LRRFIP1 as a novel protein regulating platelet function. Blood. 2010; 116:4646–4656. [PubMed: 20833976]

75. Gieger C, Radhakrishnan A, Cvejic A, Tang W, Porcu E, Pistis G, Serbanovic-Canic J, Elling U, Goodall AH, Labrune Y, et al. New gene functions in megakaryopoiesis and platelet formation. Nature. 2011; 480:201–208. [PubMed: 22139419]