Illustrated Review

Fishing for answers to hemostatic and thrombotic disease: Genome editing in zebrafish

Azhwar Raghunath PhD1 | Allison C. Ferguson BS1 | Jordan A. Shavit MD, PhD1,2

1Department of Pediatrics, University of Michigan School of Medicine, Ann Arbor, Michigan, USA
2Department of Human Genetics, University of Michigan School of Medicine, Ann Arbor, Michigan, USA

Correspondence
Jordan A. Shavit and Azhwar Raghunath, Department of Pediatrics, University of Michigan, Room 8301, Medical Science Research Building III, 1150 West Medical Center Dr, Ann Arbor, MI 48109, USA. Email: jshavit@umich.edu and razh@umich.edu

Funding Information
National Heart, Lung, and Blood Institute, Grant/Award Number: R35HL150784; National Hemophilia Foundation, Grant/Award Number: Judith Graham Pool Postdoctoral Fellowship; National Institute of Environmental Health Sciences, Grant/Award Number: R01ES032255

Handling Editor: Dr Michelle Sholzberg

Abstract
Over the past two decades, the teleost vertebrate Danio rerio (zebrafish) has emerged as a model for hemostasis and thrombosis. At genomic and functional levels, there is a high degree of conservation of the hemostatic system with that of mammals. Numerous features of the fish model offer unique advantages for investigating hemostasis and thrombosis. These include high fecundity, rapid and external development, optical transparency, and extensive functional homology with mammalian hemostasis and thrombosis. Zebrafish are particularly suited to genome-wide mutagenesis experiments for the study of modifier genes. They are also amenable to whole-organism small-molecule screens, a feature that is exceptionally relevant to hemostasis and thrombosis. Zebrafish coagulation factor knockouts that are in utero or neonatal lethal in mammals survive into adulthood before succumbing to hemorrhage or thrombosis, enabling studies not possible in mammals. In this illustrated review, we outline how zebrafish have been employed for the study of hemostasis and thrombosis using modern genome editing techniques, coagulation assays in larvae, and in vivo evaluation of patient-specific variants to infer causality and demonstrate pathogenicity. Zebrafish hemostasis and thrombosis models will continue to serve as a clinically directed basic research tool and powerful alternative to mammals for the development of new diagnostic markers and novel therapeutics for coagulation disorders through high-throughput genetic and small-molecule studies.

Keywords
coagulation, genetics, genome editing, hemostasis, thrombosis, zebrafish
Fishing for answers to hemostatic and thrombotic disease: genome editing in zebrafish

Introduction to zebrafish

The zebrafish (Danio rerio), has numerous features that make it amenable to the study of hemostasis and thrombosis, including high fecundity, rapid and external development, optical transparency, and extensive functional and genomic homology with mammals.

Technologies for genetic studies in zebrafish

Zebrafish are suited to the application of modern powerful techniques for both targeted (eg, genome editing) and genome-wide genetic manipulation.

Zebrafish models of coagulation disorders

Unlike many mammalian counterparts that exhibit in utero or neonatal lethality, zebrafish coagulation factor mutants survive into early adulthood before succumbing to coagulopathy, enabling unique types of investigation.

Hemostasis/thrombosis in zebrafish

Coagulation in zebrafish shares many features with mammals. Hemostasis can be analyzed in zebrafish using an array of techniques, including vascular injury and labeling of coagulation factors and cells with fluorescent proteins.

Evaluation of patient coagulation factor mutations

Variants of uncertain significance can be studied rapidly in vivo using zebrafish models of coagulation disorders to demonstrate pathogenicity. The zebrafish is also an ideal model for the development of new diagnostic markers and novel therapeutics for coagulation disorders. Zebrafish hemostasis and thrombosis models will continue to serve as a clinically directed basic research model and powerful alternative to mammals.

Graphical Abstract

This graphical abstract outlines our review of the use of zebrafish to study disorders of hemostasis and thrombosis. We initially introduce the zebrafish and its associated technologies. This is followed by a review of the models of hemostatic and thrombotic disease that have been produced using genome editing. Finally, we review application of zebrafish to studies of patient coagulation factor variation.

Essentials

- The zebrafish hemostatic system is highly conserved with mammals genomically and functionally.
- Hemostatic disease models produced using genome editing show conservation with human disorders.
- Zebrafish coagulation factor mutants that are early lethal in mammals survive into adulthood.
- Potential disease-causing variants can be rapidly assessed in vivo using zebrafish knockouts.
Unique and relevant features of zebrafish for the study of hemostasis and thrombosis

- Development is external, rapid, and transparent. The first week of life is sustained through yolk-based nutrition. Hundreds of larvae can be maintained in petri dishes at low cost.

- Virtually all known coagulation factors have been identified in the sequenced genome. Hemostasis and thrombosis have been shown to be functionally conserved.

- Genetically edited models are easy and cheap to produce. Fluorescently tagged proteins and structures (e.g., fibrin(ogen) and blood cells) enable visual assessment of disease states.

- ~30% of the zebrafish genome is duplicated, including some coagulation factors. Duplications can reveal subfunctionalized roles not identified in mammals.

- Larvae with engineered coagulation disorders can be arrayed into multiwell plates and assayed with small molecule libraries or used for large scale mutagenesis studies.

- Larvae with engineered coagulation disorders can be assayed with small molecule libraries or used for large scale mutagenesis studies.

- There are no known factor XI or XII orthologs in zebrafish.

- Teleost whole genome duplication after divergence from mammals.
Evolution of technology for the generation of zebrafish disease models

Germline recessive lethal and specific locus mutations are induced by exposure to ultraviolet (UV) light or gamma rays.\textsuperscript{16-18}

Zinc-finger nucleases (ZFNs) are the first successful application of genome editing to generate zebrafish disease models, but are limited in targeting, difficult to produce, and have a high failure rate.\textsuperscript{22,23}

TALEN genome editing nucleases (transcription activator-like effector) are much more efficient than ZFNs with broad targeting abilities.\textsuperscript{24,25}

CRISPR/Cas9 utilizes custom single guide RNAs (sgRNAs) that recruit a nuclease for specific targeting. These provide the efficiency and broad targeting of TALENs, but dramatically increase the ease of application.\textsuperscript{26,27}

N-ethyl-N-nitrosourea (ENU), an alkylating agent, is used for the random generation of point mutations in phenotype-driven recessive screens.\textsuperscript{19}

Chemically modified antisense oligonucleotides (morpholinos) block splicing and translation to produce gene specific knockdowns.\textsuperscript{20,21}
Patient specific variants identified in sequencing or association studies are modeled to determine pathogenicity in vivo.29

Zinc finger nucleases, TALENS, and CRISPR/Cas9 are injected into single cell embryos to target known coagulation factors.30,31

Zebrafish develop from a single cell to a free swimming larva in 3 days, at which point nearly every organ system has developed, including flowing blood and an intact coagulation system.3,5

Coagulation disorder models are used to evaluate variants of uncertain significance. Variants are engineered into expression vectors, which are then injected into single cell embryos, and tested for the ability to rescue at the larval stage.31,34,35

Disease models can be used to find modifier genes through unbiased genome-wide chemical mutagenesis15,19 (left) or novel therapeutics using small molecule libraries (right).13
Loss of p21-activated kinase 2 (Pak2−/−) in mice results in embryonic lethality secondary to developmental defects. Examination of pak2 duplication (pak2a and b) in zebrafish enabled dissection of Pak2 functions, and discovery of a role for Pak2a in vascular integrity.

Teleost fish underwent a whole genome duplication ~400 million years ago. Acquired mutations in duplicate genes can result in inactivation (nonfunctionalization), partition of multifunctional genes (subfunctionalization) or acquisition of new function (neofunctionalization). Gray boxes indicate loss of domain function.

Whole genome duplication in zebrafish has resulted in the duplication of the tf, f9, f7, and proc genes.

In zebrafish, a hypomorphic pak2a mutation causes the redhead (rhd) phenotype (intracranial hemorrhage). Only injection of pak2a mRNA rescues the rhd phenotype.

The teleost-specific genome duplication with the presence of duplicated paralogs gives a unique opportunity to identify previously undiscovered gene functions.
Zebrafish thrombocyte spreading on fibrinogen is similar to human platelets. During activation, human platelets form filopodia and lamellipodia extensions, zebrafish thrombocytes also develop pseudopod-like protrusions after activation.

**Comparison of platelets and thrombocytes**

- Anucleate with granular cytoplasm
- Produced in large numbers vastly out of proportion to nucleated blood cells
- Plasma membrane invades the platelet interior to make a surface-connected canalicular system
- Receptors mediate hemostasis and other functions
- Unique to mammals

- Nucleated with dense chromatin, cytoplasmic projections
- Relative numbers similar to white cells
- Function similar to platelets in adherence and aggregation
- Stimulated by multiple platelet agonists: eg, ADP, arachidonic acid, collagen
- Receptors for adhesion and aggregation are conserved
- Present in fish, birds, reptiles, and amphibians

**Platelet Spreading in Humans**

- Inactive
- Early activation
- Late activation
- Active

**Thrombocyte Spreading in Zebrafish**

- Inactive
- Early activation
- Late activation
- Active
Thrombosis assays in zebrafish

- Ferric chloride
  - $\text{FeCl}_3$ is layered over larvae immobilized in agarose\(^{51}\)
  - $\text{FeCl}_3$ produces free radicals causing endothelial injury and vessel occlusion
  - Time to occlusion (TTO) measured in caudal vessels

- Phenylhydrazine
  - Phenylhydrazine is layered over caudal vessels of immobilized larvae\(^{51,52}\)
  - Thought to activate flipase and externalize phosphatidylinerine in erythrocytes and thrombocytes, resulting in vessel occlusion

- Laser injury
  - Endothelium injured in agarose immobilized larvae using a pulsed nitrogen laser, resulting in specific venous or arterial vessel occlusion\(^{53}\)

Outcomes of laser-mediated endothelial injury in various transgenic lines

- Venous injury
  - Occlusive thrombi at the site of laser-mediated endothelial injury\(^{53}\)

- Arterial injury
  - Occlusive thrombi in a cd41-eGFP transgenic\(^{12}\) (green thrombocytes) shows arterial thrombi to be thrombocyte-rich, analogous to human pathology\(^{5}\)
  - Thrombocytes appear at 48 hours post fertilization (hpf)\(^{12,54}\)

- Occlusive thrombi from double cd41-eGFP and gata1-DsRed transgenics\(^{55}\) (latter labels erythrocytes red). These are fibrin-rich akin to human venous thrombosis (see fibrinogen illustration below)\(^{51,55}\) After circulation initiates at 24 hpf, primitive erythrocytes enter the vasculature\(^{56}\)

Unlike mammals, erythrocytes and thrombocytes are nucleated.
Zebrafish \textit{at}3 deficiency demonstrates overlapping and distinct phenotypes with mammals

**Mouse: embryonic lethality**
At\textit{3}-- mutants die at e16.5 due to widespread fibrin deposition in the heart and liver and paradoxical hemorrhage, implying a consumptive coagulopathy.$^{57,58}$

**Zebrafish: lethality in adulthood**
Genome editing with zinc finger nucleases was used to produce \textit{at}3 null mutants. \textit{at}3-- juveniles tolerate spontaneous thrombosis and uncontrolled disseminated intravascular coagulopathy early in development, but adults succumb to intracardiac thrombosis. Some adults exhibit visible hemorrhage (arrow).$^{59}$

**Zebrafish: spontaneous thrombosis**
\textit{at}3-- mutant zebrafish larvae labeled with fluorescently-tagged fibrinogen reveal fibrin deposition consistent with thrombosis secondary to disseminated intravascular coagulation.$^{59}$

**Zebrafish: consumptive coagulopathy**
Disseminated intravascular coagulation with consumption of fibrinogen results in the absence of occlusion following laser-mediated venous endothelial injury in \textit{at}3-- larvae at 3 dpf.$^{59}$

**Human and zebrafish \textit{at}3 cDNA transgene**
Both human and zebrafish \textit{at}3 cDNA injected into \textit{at}3-- single-cell embryos rescue the delayed occlusion after laser-mediated venous endothelial injury.$^{59}$
Common pathway gene knockout in zebrafish results in unexpected survival

Loss of $f_2$, $f_5$, and $f_{10}$ in fish are compatible with development to adulthood

Based on analysis of the zebrafish common pathway mutants, angiogenesis and vasculogenesis appear to be normal. These data suggest that thrombin generation does not play a role in these processes, and in utero death in mammals is likely due to hemorrhage.

Genome editing with TALENs and CRISPR/Cas9 was used to produce $f_2$, $f_5$, and $f_{10}$ mutant embryos/larvae, which show no overt hemorrhage. They develop normally into the mid-juvenile stage but exhibit spontaneous hemorrhage beginning around 2 months of age, with complete lethality by 4 months.\(^{31,34,60}\)

In zebrafish, common pathway knockouts survive into early adulthood before succumbing to lethal hemorrhage.

Common pathway mouse knockouts exhibit \textit{in utero} lethality due to hemorrhage versus possible vascular defects.\(^{61-65}\)
The role of fibrinogen in zebrafish hemostasis

Loss of fibrinogen results in hemorrhage in multiple tissues

*fga* mutants exhibit spontaneous hemorrhage in the jaw, abdomen, muscle, and fin, as well as the forebrain, midbrain, and hindbrain (inset).\(^{35,66}\) Hemorrhage occurs later than common pathway mutations, with partial lethality by one year of age.

Fibrin(ogen) incorporates into a developing clot in zebrafish larvae

Fluorescently tagged fibrinogen demonstrates that fibrin is the primary component of thrombi produced through endothelial injury in the venous circulation.\(^{11}\)

**fga** mutant larval fish exhibit a hemostatic defect

As in mammals, targeted mutation of *fga* results in loss of fibrinogen, and absence of thrombus formation following endothelial injury.\(^{35,67}\) This can be rescued by the expression of zebrafish *fga* cDNA or human fibrinogen infusion. Surprisingly, incubation with \(\varepsilon\)-aminocaproic acid also partially reverses this hemostatic defect.\(^{35}\)
Loss of nuclear factor erythroid 2 (Nfe2) results in defective adult but normal embryonic/larval thrombopoiesis in zebrafish

Mouse: thrombocytopenia and neonatal hemorrhage
Nfe2\(^{-/-}\) mutants develop normally except for severe, nearly absolute thrombocytopenia. Most homozygous mutants die in the neonatal period secondary to hemorrhage.\(^{68}\)

Zebrafish: nfe2\(^{-/-}\) larvae exhibit normal thrombocyte function
TALENs were used to engineer a null mutation in nfe2.\(^{49}\) Homozygous mutants display normal thrombocyte-rich clots (arrow, right) in the arterial circulation following laser-mediated endothelial injury of the dorsal aorta at 6 dpf.

Zebrafish: larval thrombopoiesis
nfe2\(^{-/-}\) mutant larva are indistinguishable from wild type siblings. They have normal numbers of thrombocytes and aggregate in response to laser-mediated arterial endothelial injury.\(^{49}\)

Zebrafish: adult thrombopoiesis
nfe2\(^{-/-}\) adults display severe thrombocytopenia, but no apparent hemorrhage and normal long-term survival. Mutant thrombocytes have demonstrably decreased spreading on fibrinogen.\(^{49}\)
adamts13 and vwf in zebrafish

Wild-type zebrafish

VWF
Thrombocyte
Endothelial cell
ADAMTS13
Cleaves large multimers of VWF
Weibel-Palade body
lysine-rich histone

Normal Blood Flow

adamts13/- zebrasfish

VWF
Neutrophil
No cleavage of VWF
Endothelial cell

Thrombotic thrombocytopenic purpura

Adams13 deficiency alone does not cause acute thrombotic thrombocytopenic purpura
In adamts13/- zebrafish mutants, intraperitoneal injection of lysine-rich histone triggers severe thrombotic thrombocytopenic purpura (TTP) and causes death. This is mediated through Vwf, as adamts13/-;vwf/- zebrafish mutants do not develop TTP.

Validated fish antibodies are scant. Zheng et al. had to produce their own anti-Vwf antibody to visualize multimers.

Vwf in zebrafish: zebrafish Vwf forms high molecular multimers and is packaged into Weibel-Palade bodies. Loss of Vwf results in a higher time to occlusion in vwf/- mutant fish than the wild type larvae in both the arterial and venous circulation.
Complete loss of common pathway factors is embryonic lethal in mouse models and presumed lethal in humans.\textsuperscript{31,34,60} Hypotheses for the differences between mammals and zebrafish include:

- absence of birth trauma
- differential hemostatic challenges in an aquatic environment
- lower blood pressures in zebrafish
- species-specific genetic differences resulting in a differential baseline hemostatic balance\textsuperscript{60,72}

(Black and red lines indicate average age of death for each mutant line)
### Evaluation of human genetic variants in zebrafish hemostasis and thrombosis knockout models

#### AT3 thrombosis variants

| Variant       | Affected domain(s)                                      | Ability to rescue at3 mutant DIC phenotype |
|---------------|--------------------------------------------------------|-------------------------------------------|
| Leu99Phe      | heparin binding domain                                 | complete                                  |
| Lys114Glu     | heparin binding domain                                 | complete                                  |
| Arg393His     | reactive center loop (RCL) : P1 Arg mutation           | none                                      |
| Ala404Asp     | pleiotropic effect on RCL and heparin binding domain   | partial                                   |

#### F5 deficiency variants

| Variant       | Affected domain(s) | Ability to rescue f5 mutant phenotype |
|---------------|--------------------|--------------------------------------|
| Ser83Arg      | A1                 | complete                             |
| Gly97Asp      | A1                 | none                                 |
| Tyr1702Cys    | A3                 | none                                 |
| Arg2074Cys    | C2                 | complete                             |
| Arg2187Cys    | C2                 | complete                             |

#### F10 deficiency variants

| Variant       | Affected domain(s) | Ability to rescue f10 mutant phenotype |
|---------------|--------------------|---------------------------------------|
| Arg68Cys      | EGF1               | none                                  |
| Gly173Typ     | activation peptide | none                                  |
| ΔT176_Q186 deletion | activation peptide | none                                  |
| Gly262Asp     | protease           | none                                  |
| Ile323Met     | protease           | partial                               |
| Cys390Phe     | protease           | none                                  |
| Gln416Leu     | protease           | partial                               |

#### FGA variants

| Variant       | Affected domain(s) | Ability to rescue fga mutant phenotype |
|---------------|--------------------|---------------------------------------|
| Met1Val       | pre-peptide        | none                                  |
| Cys55Gly      | N-terminus         | complete                              |
| Cys64Tyr      | N-terminus         | complete                              |
| Tyr809Cys     | αEC domain         | none                                  |

Since hemostasis must occur within a closed circulatory system, consisting of flowing blood, proteins, cells, and endothelium, the zebrafish is superior to cell culture systems for assessing variants.
AUTHOR CONTRIBUTIONS
AR and JS developed the concepts and wrote the manuscript. AR and AF produced the illustrations. AR, AF, and JS approved the final content.

ACKNOWLEDGMENTS
The authors thank members of the Shavit laboratory for helpful comments and suggestions.

FUNDING INFORMATION
This work was supported by the National Hemophilia Foundation Judith Graham Pool Postdoctoral Fellowship Award (AR) and National Institutes of Health grants R35HL150784 and R01ES032255 (JAS). JAS is the Henry and Mala Dorfman Family Professor of Pediatric Hematology/Oncology.

RELATIONSHIP DISCLOSURE
JAS has been a consultant for Sanofi, Takeda, CSL Behring, HEMA Biologics, and Bayer. AR and ACF report no conflicts of interest.

ORCID
Azhwar Raghunath https://orcid.org/0000-0001-5217-0341
Allison C. Ferguson https://orcid.org/0000-0003-3195-8572
Jordan A. Shavit https://orcid.org/0000-0002-2874-4904

TWITTER
Azhwar Raghunath @raghugreek
Jordan A. Shavit @Clot1

REFERENCES
1. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn. 1995;203(3):253-310.
2. Matthews M, Trewarva B, Matthews J. A virtual tour of the guide for zebrafish users. Lab Anim (NY). 2002;31(3):34-40.
3. Isogai S, Horiguchi M, Weinstein BM. The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. Dev Biol. 2001;230(2):278-301.
4. Weyand AC, Shavit JA. Zebrafish as a model system for the study of hemostasis and thrombosis. Curr Opin Hematol. 2014;21(5):418-422.
5. Rost MS, Grzegorski SJ, Shavit JA. Quantitative methods for studying hemostasis in zebrafish larvae. Methods Cell Biol. 2016;134:377-389.
6. Howe K, Clark MD, Torroja CF, et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 2013;496(7446):498-503.
7. Hanumanthaiya R, Day K, Jagadeeswaran P. Comprehensive analysis of blood coagulation pathways in teleosts: evolution of coagulation factor genes and identification of zebrafish factor VIII. Blood Cells Mol Dis. 2002;29(1):57-68.
8. Meyer A, Scharl M. Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. Curr Opin Cell Biol. 1999;11(6):699-704.
9. Jagadeeswaran P, Cooley BC, Gross PL, Mackman N. Animal models of thrombosis from zebrafish to nonhuman primates: use in the elucidation of new pathologic pathways and the development of antithrombotic drugs. Circ Res. 2016;118(9):1363-1379.
10. Rafferty SA, Quinn TA. A beginner’s guide to understanding and implementing the genetic modification of zebrafish. Prog Biophys Mol Biol. 2018;138:3-19.
11. 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(9):e74682.
12. Lin HF, Traver D, Zhu H, et al. Analysis of thrombocyte development in CD41-GFP transgenic zebrafish. Blood. 2005;106(12):3803-3810.
13. Lam PY, Peterson RT. Developing zebrafish disease models for in vivo small molecule screens. Curr Opin Chem Biol. 2019;50:37-44.
14. Patton EE, Zon LI, Langenau DM. Zebrafish disease models in drug discovery: from preclinical modelling to clinical trials. Nat Rev Drug Discov. 2021;20(8):611-628.
15. Santoriello C, Zon LI. Hooked! Modeling human disease in zebrafish. J Clin Invest. 2012;122(7):2337-2343.
16. Walker C, Streisinger G. Induction of mutations by gamma-rays in Pregonial germ cells of zebrafish embryos. Genetics. 1983;103(1):125-136.
17. Chakrabarti S, Streisinger G, Singer F, Walker C. Frequency of gamma-ray induced specific locus and recessive lethal mutations in mature germ cells of the zebrafish, Brachydanio rerio. Genetics. 1983;103(1):109-123.
18. Streisinger G, Walker C, Dower N, Knauber D, Singer F. Production of clones of homozygous diploid zebrafish (Brachydanio rerio). Nature. 1981;291(5813):293-296.
19. Grosveld G, Nusse R. Development of the zebrafish and telomerase. Science. 1991;252(5003):807-811.
20. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn. 1995;203(3):253-310.
21. Matthews M, Trewarva B, Matthews J. A virtual tour of the guide for zebrafish users. Lab Anim (NY). 2002;31(3):34-40.
22. Isogai S, Horiguchi M, Weinstein BM. The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. Dev Biol. 2001;230(2):278-301.
23. Weyand AC, Shavit JA. Zebrafish as a model system for the study of hemostasis and thrombosis. Curr Opin Hematol. 2014;21(5):418-422.
24. Rost MS, Grzegorski SJ, Shavit JA. Quantitative methods for studying hemostasis in zebrafish larvae. Methods Cell Biol. 2016;134:377-389.
25. Howe K, Clark MD, Torroja CF, et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 2013;496(7446):498-503.
26. Hanumanthaiya R, Day K, Jagadeeswaran P. Comprehensive analysis of blood coagulation pathways in teleosts: evolution of coagulation factor genes and identification of zebrafish factor VIII. Blood Cells Mol Dis. 2002;29(1):57-68.
27. Meyer A, Scharl M. Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. Curr Opin Cell Biol. 1999;11(6):699-704.
28. Jagadeeswaran P, Cooley BC, Gross PL, Mackman N. Animal models of thrombosis from zebrafish to nonhuman primates: use in the elucidation of new pathologic pathways and the development of antithrombotic drugs. Circ Res. 2016;118(9):1363-1379.
29. Rafferty SA, Quinn TA. A beginner’s guide to understanding and implementing the genetic modification of zebrafish. Prog Biophys Mol Biol. 2018;138:3-19.
30. 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(9):e74682.
31. Lin HF, Traver D, Zhu H, et al. Analysis of thrombocyte development in CD41-GFP transgenic zebrafish. Blood. 2005;106(12):3803-3810.
32. Lam PY, Peterson RT. Developing zebrafish disease models for in vivo small molecule screens. Curr Opin Chem Biol. 2019;50:37-44.
33. Patton EE, Zon LI, Langenau DM. Zebrafish disease models in drug discovery: from preclinical modelling to clinical trials. Nat Rev Drug Discov. 2021;20(8):611-628.
34. Santoriello C, Zon LI. Hooked! Modeling human disease in zebrafish. J Clin Invest. 2012;122(7):2337-2343.
35. Walker C, Streisinger G. Induction of mutations by gamma-rays in Pregonial germ cells of zebrafish embryos. Genetics. 1983;103(1):125-136.
36. Chakrabarti S, Streisinger G, Singer F, Walker C. Frequency of gamma-ray induced specific locus and recessive lethal mutations in mature germ cells of the zebrafish, Brachydanio rerio. Genetics. 1983;103(1):109-123.
37. Streisinger G, Walker C, Dower N, Knauber D, Singer F. Production of clones of homozygous diploid zebrafish (Brachydanio rerio). Nature. 1981;291(5813):293-296.
38. Grosveld G, Nusse R. Development of the zebrafish and telomerase. Science. 1991;252(5003):807-811.
33. Wei X, Ju X, Yi X, et al. Identification of sequence variants in genetic disease-causing genes using targeted next-generation sequencing. PLoS One. 2011;6(12):e29500.

34. Hu Z, Liu Y, Huanng MC, et al. Genome editing of factor X in zebrafish reveals unexpected tolerance of severe defects in the common pathway. Blood. 2017;130(5):666-676.

35. Hu Z, Lavik KI, Liu Y, et al. Loss of fibrinogen in zebrafish results in an asymptomatic embryonic hemostatic defect and synthetic lethality with thrombocytopenia. J Thromb Haemost. 2019;17(4):607-617.

36. Glasauer SM, Neuhauss SC. Whole-genome duplication in teleost fishes and its evolutionary consequences. Mol Genet Genomics. 2014;289(6):1045-1060.

37. Braasch I, Bobe J, Guiguen Y, Postlethwait JH. Reply to: 'Subfunctionalization versus neo-functionalization after whole-genome duplication'. Nat Genet. 2018;50(7):910-911.

38. Iyer N, Al Qaryoute A, Kacham M, Jagadeeswaran P. Identification of zebrafish ortholog for human coagulation factor IX and its age-dependent expression. J Thromb Haemost. 2021;19(9):2137-2150.

39. Buchner DA, Su F, Yamaoka JS, et al. pak2a mutations cause cerebral hemorrhage in redhead zebrafish. Proc Natl Acad Sci USA. 2007;104(35):13996-14001.

40. Arias-Romero LE, Chernoff J. A tale of two paks. Bioll Cell. 2008;100(2):97-108.

41. Thon JN, Italiano JE. Platelets: production, morphology and ultrastructure. Handb Exp Pharmacol. 2012;210:3-22.

42. Lee D, Fong KP, King MR, Brass LF, Hammer DA. Differential dynamics of platelet contact and spreading. Biophys J. 2012;102(3):472-482.

43. Hartwig JH. Mechanisms of actin rearrangements mediating platelet activation. J Cell Biol. 1992;118(6):1421-1442.

44. Khandekar G, Kim S, Jagadeeswaran P. Zebrafish thrombocytes: functions and origins. Adv Hematol. 2012;2012:857058.

45. Jagadeeswaran P, Sheehan JP, Craig FE, Troyer D. Identification and characterization of zebrafish thrombocytes. Br J Haematol. 1999;107(4):731-738.

46. Lloyd-Evans P, Barrow SE, Hill DJ, et al. Eicosanoid generation and effects on the aggregation of thrombocytes from the rainbow trout, Oncorhynchus mykiss. Biochim Biophys Acta. 1994;1215(3):291-299.

47. Hill DJ, Hallett MB, Rowley AF. Effect of prostanooids and their precursors on the aggregation of rainbow trout thrombocytes. Am J Physiol. 1999;276(3):R659-R664.

48. Hughes CE, Radhakrishnan UP, Lordkipanidze M, et al. G6f-like is an ITAM-containing collagen receptor in thrombocytes. PLoS One. 2012;7(12):e52622.

49. Rost MS, Shetstopolov I, Liu Y, et al. Nfe2 is dispensable for early but required for adult thromocyte formation and function in zebrafish. Blood Adv. 2018;2(23):3418-3427.

50. Sandmann R, Köster S. Topographic cues reveal two distinct spreading mechanisms in blood platelets. Sci Rep. 2016;6:22357.

51. Gregory M, Hanumanthaih R, Jagadeeswaran P. Genetic analysis of hemostasis and thrombosis using vascular occlusion. Blood Cells Mol Dis. 2002;29(3):286-295.

52. Zhu XY, Liu HC, Guo SY, et al. A zebrafish thrombosis model for assessing antithrombotic drugs. Zebrafish. 2016;13(4):335-344.

53. Jagadeeswaran P, Carrillo M, Radhakrishnan UP, Rajpurukh SH, Kim S. Laser-induced thrombosis in zebrafish. Methods Cell Biol. 2011;101:197-203.

54. Huang MC, Shavit JA. Simple and rapid quantification of thrombocytes in zebrafish larvae. Zebrafish. 2015;12(3):238-242.

55. Traver D, Paw BH, Poss KD, Penberthy WT, Lin S, Zon LI. Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. Nat Immunol. 2003;4(12):1238-1246.

56. Long Q, Meng A, Wang H, Jessen JR, Farrell MJ, Lin S. GATA-1 expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene. Development. 1997;124(20):4105-4111.

57. Ishiguro K, Kojima T, Kadomatsu K, et al. Complete antithrombin deficiency in mice results in embryonic lethality. J Clin Invest. 2000;106(7):873-878.

58. Kojima T. Targeted gene disruption of natural anticoagulant proteins in mice. Int J Hematol. 2002;76(Suppl 2):36-39.

59. Liu Y, Kretz CA, Maeder ML, et al. Targeted mutagenesis of zebrafish antithrombin III triggers disseminated intravascular coagulation and thrombosis, revealing insight into function. Blood. 2014;124(1):142-150.

60. Grzegorski SJ, Hu Z, Liu Y, et al. Disruption of the kringle 1 domain of prothrombin leads to late onset mortality in zebrafish. Sci Rep. 2020;10(1):4049.

61. Cui J, O’Shea KS, Purkayastha A, Saunders TL, Ginsburg D. Fatal haemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V. Nature. 1996;384(6604):66-68.

62. Sun WY, Witte DP, Degen JL, et al. Prothrombin deficiency results in embryonic and neonatal lethality in mice. Proc Natl Acad Sci USA. 1998;95(13):7597-7602.

63. Xue J, Wu Q, Westfield LA, et al. Incomplete embryonic lethality and fatal neonatal hemorrhage caused by prothrombin deficiency in mice. Proc Natl Acad Sci USA. 1998;95(13):7603-7607.

64. Dewerchin M, Liang Z, Moons L, et al. Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice. Thromb Haemost. 2000;83(2):185-190.

65. Mullins ES, Kombrinck KW, Talmage KE, et al. Genetic elimination of prothrombin in adult mice is not compatible with survival and results in spontaneous hemorrhagic events in both heart and brain. Blood. 2009;113(6):696-704.

66. Fish RJ, Di Sanza C, Neerman-Arbez M. Targeted mutation of zebrafish fga models human congenital afibrinogenemia. Blood. 2014;123(14):2278-2281.

67. Fish RJ, Freire C, Di Sanza C, Neerman-Arbez M. Venous thrombosis and thrombocyte activity in zebrafish models of quantitative and qualitative fibrinogen disorders. Int J Mol Sci. 2021;22(2):655.

68. Shvidasani RA, Rosenblatt MF, Zucker-Franklin D, et al. Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MGDF in megakaryocyte development. Cell. 1995;81(5):695-704.

69. Zheng L, Abdelgawwad MS, Zhang D, et al. Histone-induced thrombotic thrombocytopenic purpura in adams13 (~1) zebrafish depends on von Willebrand factor. Haematologica. 2020;105(4):1107-1119.

70. Ghosh A, Vo A, Twiss BK, et al. Characterization of zebrafish von Willebrand factor reveals conservation of domain structure, multimerization, and intracellular storage. Adv Hematol. 2012;2012:214209.

71. Iyer N, Tcheuyap VT, Schneider S, Marshall V, Jagadeeswaran P. Knockout of von Willebrand factor in zebrafish by CRISPR/Cas9 mutagenesis. Br J Haematol. 2019;186(4):e76-e80.

72. Shavit JA, Ginsburg D. Hemophilias and other disorders of hemostasis. In: Rimoin DL, Pyeritz RE, Korf BR, eds. Emery and Rimoin’s Principles and Practice of Medical Genetics. 6th ed. Elsevier Science; 2013:1-33.

73. Olds RJ, Lane DA, Boisclair M, Sas G, Bock SC, Thein SL. Antithrombin Budapest 3. An antithrombin variant with reduced heparin affinity resulting from the substitution L99F to Phe mutation and childhood thromboembolism. Thromb Haemost. 2001;86(4):1007-1011.

74. Mushunjhe A, Zhou A, Huntington JA, Conard J, Carrell RW. Antithrombin ‘DREUX’ (Lys 114Glu): a variant with complete loss of heparin affinity. Thromb Haemost. 2002;88(3):436-443.
76. Picard V, Susen S, Bellucci S, Aiach M, Alhenc-Gelas M. Two new antithrombin variants support a role for K114 and R13 in heparin binding. *J Thromb Haemost*. 2003;1(2):386-387.
77. Bauer KA, Ashenhurst JB, Chediak J, Rosenberg RD. Antithrombin "Chicago": a functionally abnormal molecule with increased heparin affinity causing familial thrombophilia. *Blood*. 1983;62(6):1242-1250.
78. Owen MC, Beresford CH, Carrell RW. Antithrombin Glasgow, 393 Arg to his: a P1 reactive site variant with increased heparin affinity but no thrombin inhibitory activity. *FEBS Lett*. 1988;231(2):317-320.
79. Luxembourg B, Delev D, Geisen C, et al. Molecular basis of antithrombin deficiency. *Thromb Haemost*. 2011;105(4):635-646.
80. Castoldi E, Lunghi B, Minguozzi F, et al. A missense mutation (Y1702C) in the coagulation factor V gene is a frequent cause of factor V deficiency in the Italian population. *Haematologica*. 2001;86(6):629-633.
81. Duga S, Montefusco MC, Asselta R, et al. Arg2074Cys missense mutation in the C2 domain of factor V causing moderately severe factor V deficiency: molecular characterization by expression of the recombinant protein. *Blood*. 2003;101(1):173-177.
82. Smith N, Bornikova L, Noetzli L, et al. Identification and characterization of novel mutations implicated in congenital fibrinogen disorders. *Res Pract Thromb Haemost*. 2018;2(4):800-811.
83. Zhang JZ, Redman CM. Role of interchain disulfide bonds on the assembly and secretion of human fibrinogen. *J Biol Chem*. 1994;269(1):652-658.
84. Hanss M, Pouymayou C, Blouch MT, et al. The natural occurrence of human fibrinogen variants disrupting inter-chain disulfide bonds ([alpha]Cys36Gly, [alpha]Cys36Arg and [alpha]Cys45Tyr) confirms the role of N-terminal [alpha] disulfide bonds in protein assembly and secretion. *Haematologica*. 2011;96(8):1226-1230.
85. https://varsome.com/variant/hg19/

How to cite this article: Raghunath A, Ferguson AC, Shavit JA. Fishing for answers to hemostatic and thrombotic disease: Genome editing in zebrafish. *Res Pract Thromb Haemost*. 2022;6:e12759. doi: 10.1002/rth2.12759