Zebrafish for thrombocytopoiesis- and hemostasis-related researches and disorders

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Abstract

Platelets play vital roles in hemostasis, inflammation, and vascular biology. Platelets are also active participants in the immune responses. As vertebrates, zebrafish have a highly conserved hematopoietic system in the developmental, cellular, functional, biochemical, and genetic levels with mammals. Thrombocytes in zebrafish are functional homologs of mammalian platelets. Here, we summarized thrombocyte development, function, and related research techniques in zebrafish, and reviewed available zebrafish models of platelet-associated disorders, including congenital amegakaryocytic thrombocytopenia, inherited thrombocytopenia, essential thrombocythemia, and blood coagulation disorders such as gray platelet syndrome. These elegant zebrafish models and methods are crucial for understanding the molecular and genetic mechanisms of thrombocyte development and function, and provide deep insights into related human disease pathophysiology and drug development.

Keywords: Animal models, Platelet-associated diseases, Thrombocytopoiesis, Zebrafish

1. INTRODUCTION

During the last few decades, zebrafish (Danio rerio) has grown as an important model organism for studying hematopoiesis and hematological diseases. Zebrafish and human share high similarity in genome and as high as 82% conservation in disease-associated genes.[1] In addition, zebrafish have specific features, including high fecundity, external fertilization, and transparency at the early stages of development. With these unique features, zebrafish embryos are extremely suitable for imaging and high-throughput genetic or drug screenings. Particularly, the zebrafish blood system is highly conserved with mammals, with comparable hematopoietic cell contents, similar developmental process, regulatory mechanisms, and pathogenesis.[2] Thus, zebrafish is an optimized organism for studying blood system development and for modeling human blood disorders.

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The platelet is an essential component of blood cells and is mainly responsible for preventing bleeding by clotting. In mammalian, platelets are anucleated cytoplasmic fragments of megakaryocytes (MKs), which differentiated from hematopoietic stem cells (HSCs).[3] HSCs give rise to common lymphoid progenitors and common myeloid progenitors, which can differentiate into granulocyte/macrophage progenitors and MKs-erythrocyte progenitors (MEPs), respectively.[4] MEPs are bipotent precursors that can produce both megakaryocytic cells and erythroid cells. The hematopoiesis process is highly conserved between zebrafish and mammals. Zebrafish HSCs originated in the ventral wall of the aorta, the mammalian aorta gonad mesonephros equivalents, and eventually seed the pronephros/kidney that is analogous to the bone marrow in mammals.[5] In zebrafish, thrombocytes are the mammalian platelet equivalents.[6] They appear in circulation at around 36 hpf,[7] and also are derived from the thrombocyte and erythroid progenitors,[8] the mammalian MEP equivalents, indicating the thrombocyte origin and lineage conservation between zebrafish and mammals.

In mammals, the developmental process for generating MKs is tightly controlled by several transcription factors. For example, GATA1 and FLI1 can bind the enhancers of multiple MK-specific genes and promote MEPs to differentiate into MKs.[9,10] Besides transcription factors, cytokines are also critical for thrombocytopoiesis. Thrombopoietin (TPO) is a critical hematopoietic cytokine that can regulate the MK progenitor expansion and differentiation.[11] The proliferation and maturation of MK progenitor cells depend on the circulating TPO by binding to the MPL receptor.[12] Once TPO binds to MPL on the surface of the target cell, MPL dimerization occurs and the downstream tyrosine phosphorylation pathway is activated.[13] The Janus kinase (JAK)/signal transducer and activation of transcription (STAT) pathway is one of the most important pathways for

Table 1

Summary of zebrafish models for thrombopoiesis and thrombosis disorders.

Disorders	Related gene	Method	Phenotype
Congenital amegakaryocytic thrombocytopenia (CAMT)	*mpl*	Mutation	Severe thrombocytopenia, bleeding tendency[17]
Inherited thrombocytopenia (IT)	*ptprj*	Mutation	Small platelets, migration defects[56]
	nfe2	Mutation	Thrombocytes severe reduced, function decreased[65]
Essential thrombocythemia (ET)	*calr*	Human mRNA overexpression	Thrombocytosis, HSC increased[81]
	jak2a^{V581F}	mRNA overexpression	Thrombocytosis, erythrocytosis[17,75]
Blood coagulation disorders			
Gray platelet syndrome (GPS)	*nbeal2*	Morpholino knockdown	Mild to moderate bleeding tendency[83]
Thrombocyte function-related genes	*gpr34l*	Mutation	Thrombocyte aggregation defect[90]

thrombocytic cell expansion and differentiation, as STATs are finally activated by JAK2 kinase (JAK2) phosphorylation.[13–15] In zebrafish thrombocytopoiesis, intrinsic pathways mediated by transcription factors and the extrinsic pathway induced by TPO are also conserved with mammals.[16,17]

The well-known feature of platelets is the contribution to hemostasis and thrombosis.[18] In mammalian, following the endothelial injury, the platelets are recruited to the site of injury and captured by platelet adhesive receptors such as glycoprotein (GP) Ib/IX interacted with collagen and von Willebrand factor (vWF).[19,20] Platelet activation can also be induced by the coagulants such as thrombin, ADP, and thrombin A2 (TXA2) by binding with the protease-activated receptor/ADP receptors, respectively.[21,22] Subsequently, the activation stimulates the platelet release reaction, such as the secretion of platelet α-granules and dense granule contents. Finally, the initial platelet plug is formed to seal the injured vessel.[23] Once "platelet plug" is formed, coagulation factors induce a burst of thrombin production, and soluble fibrinogen converts to an insoluble fibrin clot that can format the fibrin mesh and strengthens the thrombus.[23,24] Like mammals, zebrafish have a similar coagulation cascade, as their thrombocytes express the fibrinogen receptor GPIb (the vWF receptor) and can aggregate after stimulation with collagens, ADP, and vWF.[6,7] Moreover, the thrombin receptors, protease-activated receptor, and the ADP receptors present on the zebrafish thrombocytes membrane.[25,26] Thus, zebrafish have conserved hemostatic pathways in platelet function and coagulation.

The similarities between zebrafish and mammals in thrombocyte developmental and physiological processes indicate that the research progresses on zebrafish will shed light on the understanding of human platelet-related biology and diseases. The unique features of zebrafish, including the transparent embryos, coupled with fluorescent protein labeling methods permit it easy to visualize the interactions of thrombocyte cells and other components in vivo. The high fecundity and external fertilization make the zebrafish become a suitable model vertebrate in high-throughput studies on thrombocytopoiesis, hemostasis, thrombosis, and modeling human platelet-related diseases. Taking advantage of the zebrafish thrombocyte-specific transgenic lines such as *Tg*(*cd41l:eGFP*)[27] and *Tg*(*mpl:eGFP*),[17] combined with laser-induced injury technology and thrombocyte function evaluation approaches,[28] more thrombocyte-related genes can be identified through functional screens, which will provide new insights for human platelet-related disorders. Here, we summarize recently developed methods for studying thrombocytes in zebrafish and review currently available zebrafish mutants for thrombocytic deficiencies and models for thrombocyte-associated disorders in detail (Table 1).

2. METHODS IN ZEBRAFISH FOR HEMOSTASIS AND COAGULATION RESEARCH

2.1. Thrombocyte labeling

Selective labeling of zebrafish thrombocytes is important for tracing and isolating thrombocytic cells. In this case, the developmental, physiological, and pathogenetic changes of thrombocytes could be observed in vivo.

DiI-C_{18}, a lipophilic fluorescent dye, has been shown to label thrombocytes in live zebrafish.[7] Through injecting DiI-C_{18} into zebrafish, young thrombocytes with active function and high adhesion ability were marked with DiI-C_{18}.[29]

For stably labeling thrombocytes in zebrafish, one approach is to generate transgenic lines expressing fluorescent genes driven by a lineage-specific-promoter. CD41 is clearly expressed on the surface of platelets, MK/platelet progenitors, and early hematopoietic progenitors.[30–32] *Tg*(*cd41:eGFP*) transgenic fish line is widely used for labeling HSPCs (GFP^{low}) and thrombocytes (GFP^{high}) from embryonic stages to adulthood.[27] The transcription factor GATA1 is a master regulator for erythropoiesis and thrombocytopoiesis.[33,34] The zebrafish G1-GM2 line expresses GFP driven by GATA1 promoter, so erythrocytes and thrombocytes both are labeled in this transgenic line.[16,35] The ETS family transcription factor FLI1 begins its expression in endothelial cells and interacts with GATA1 for both erythroid and megakaryocytic differentiation.[9] *Tg*(*fli1:eGFP*) y1 lines could label endothelial cells as expected, and it also marks thrombocytes selectively.[16] Zebrafish *mpl*, the Tpo receptor, has been shown to be conserved with mammals,[27] and its expression is restricted in thrombocyte lineage.[17] The *Tg*(*mpl:eGFP*) line predominantly (~90%) marks thrombocytes and thrombocyte precursors in embryonic and larval stages.[17] Zebrafish *mylpfa* (previous names *mlc2* and *mylz2*) is a homolog for human myosin light chain 2 *MLC2* (*MYL2*) gene. MLC2 protein is essential for mammalian platelet function,[36] and the MLC kinase and its phosphorylation in thrombocytes are conserved in zebrafish.[37] Recently, Glo fish *Tg*(*mylz2:RFP*), a transgenic line with *mylz2* promoter-driven RFP gene, has been generated and reported to label DiI-C_{18}^{+} young thrombocytes in zebrafish.[38] They also generated GloFli (Glo fish crossed with *Tg*(*fli1:eGFP*)) in which DiI-C_{18}^{+} and DiI-C_{18}^{-} thrombocytes are labeled with RFP and GFP, respectively.[38]

These findings are definitely helpful in the identification of developing and functional thrombocytes and provide useful tools for the study of thrombocytopoiesis in zebrafish. Although achievements of thrombocyte labeling have been made in zebrafish, it is important to distinguish more diverse thrombocytes, such as apoptotic-related thrombocytes, immune response-related thrombocytes, and thrombocytes in specific developmental stages.

2.2. Thrombocyte quantification in zebrafish

The platelet count is an intuitionistic parameter to reflect the abundance of platelets in an organism. To quantify thrombocytes in zebrafish, several approaches can be applied in different developmental stages of animals. Whole-mount in situ hybridization (WISH) experiments can be performed to determine gene expression patterns with thrombocytopoietic lineage-specific probes at embryonic and larval stages.[39] Lineage-specific transgenic reporter lines facilitate the quantification of thrombocytes, with fluorescent cell counts by antibody staining against fluorescent proteins or by flow cytometry analysis.[27]

2.3. Thrombocyte functional assay in zebrafish

The predominant function of thrombocytes is aggregation when stimuli or injuries occur in the blood vessels. In order to visualize thrombocytes aggregation and adhesion in vivo, several approaches are reported to introduce the injuries into the blood system:

(1) **By punching:** Embryos can be injured by puncturing the blood vessel in the tail region with a glass needle, and the bleeding status can be observed under the microscope.[40]
(2) **By chemical induction:** Vascular occlusion can be induced by specific chemicals. $FeCl_3$ is reported to cause endothelial injury by free radical production,[41] and it can induce zebrafish vascular occlusion in the caudal artery.[41,42] Besides $FeCl_3$, phenylhydrazine can also lead to arterial thrombus formation of zebrafish larvae.[42]
(3) **By laser injury:** Pulsed nitrogen laser light has previously been used to cause a vessel lesion that can cause the formation of the vascular occlusion.[43] It is very easy to use myotomes as reliable markers for repeatedly selecting the exact location of the vessel for injury in zebrafish, while it is difficult to consistently target the same vessel locations in mammalian models.[28]

To evaluate the thrombocyte activation and adhesion abilities, several parameters can be utilized in zebrafish similar to mammals. Due to the transparency of fish embryos and larvae, the thrombus formation, growth, and dissolution can be visualized in live animals under the microscope. It is accessible to detect the time to attachment, the time when the first cell adheres to the injury site. Time to attachment represents the thrombus initiation ability, including thrombocyte activation, and thrombocyte–subendothelium interactions.[44] It is also easy to calculate the time to occlusion of the vessel corresponding to the thrombus growth ability, including thrombocyte activation and aggregation.[45] In addition, the thrombus surface area can also be utilized in zebrafish for a better understanding of thrombus growth ability.[46] To monitor the thrombus stability and retraction, the time to dissolution could be recorded in zebrafish.[43] In adult zebrafish, a direct way to analyze the function of thrombocytes is to cut the caudal region and calculate the bleeding time. The aggregation of thrombocytes can also be monitored by a tilt plate assay in vitro. By observing a certain volume of blood in PBS (containing coagulants) in a tilt plate, aggregation and adhesion can be evaluated by the firm button formation and the migration speed.[6]

These approaches are undoubtedly useful for the evaluation of thrombocyte-related functions in zebrafish. Along with our developing learning on new functions of thrombocytes, new evaluation methods or parameters should be developed on time for the extensive application of zebrafish model.

3. UNDERSTANDING THROMBOCYTE-RELATED DISORDERS USING ZEBRAFISH

3.1. Thrombocytopenia

Thrombocytopenia is a kind of disorder mainly manifested as a low platelet count, characterized by easy bruising and increased bleeding risk, and the severe bleeding can become life-threatening. Thrombocytopenia caused by gene mutations can be precisely resembled in animal models. Several kinds of gene-related thrombocytopenia have been studied in zebrafish, including the following.

3.1.1. Congenital amegakaryocytic thrombocytopenia (CAMT) and the related gene MPL. CAMT is a rare bone marrow failure syndrome characterized by severe thrombocytopenia.[47] CAMT patient is characterized by reduced MKs, increased bleeding tendency, and predisposed to aplastic anemia.[47–49] Mutations in *MPL* has been demonstrated as the cause of CAMT.[50] *MPL* gene encodes for myeloproliferative leukemia protein, the receptor for TPO. TPO–MPL is the most important pathway regulating platelet in both mammals and vertebrates.[51] The transgenic c-MPL-deficient mice show significant thrombocytopenia and defective megakaryocytopoiesis[51] and a reduction in hematopoietic progenitor cells.[52] In zebrafish, knockdown of the *mpl* gene by morpholino effectively eliminated the numbers of circulating thrombocytes (*cd41*: GFP^+ cells).[27] Lin et al generated an inheritable zebrafish *mpl* mutant and showed that disruption of *mpl* led to a severe reduction of thrombocytes from embryonic stage to adulthood by proliferation defection of the thrombocyte precursors, as well as deficiencies in adult HSPCs.[17] Similar to CAMT patients, *mpl* mutation zebrafish display deficient hemostasis and increased bleeding tendency. Moreover, due to the restricted expression of *mpl* in thromboctyopoietic lineage, the transgenic reporter line *Tg*(*mpl:eGFP*) labels thrombocytes selectively. Together with *Tg*(*mpl:eGFP*) transgenic line, the CAMT model can serve as a precise model for anti-CAMT drug evaluation and screening, as well as for understanding TPO/MPL-dependent or TPO/MPL-independent thrombocytopoiesis.

3.1.2. Inherited thrombocytopenia (ITs) and zebrafish-related mutants. ITs are a group of heterogeneous disorders, with genetic mutations in genes associated with MK differentiation and/or platelet formation, characterized by a reduced platelet count and impaired hemostasis.[53] Due to extensive application of high-throughput sequencing, increasing numbers of causative genes have been revealed to result in different forms of IT.[53] Taking advantages of zebrafish gene manipulation approaches and its drug development potential, several IT-associated genes have been targeted in zebrafish for generating IT-like zebrafish models.

3.1.3. IT and PTPRJ (CD148). PTPRJ (CD148) is a receptor-like protein tyrosine phosphatase that is expressed highly in MKs and platelets in human. PTPRJ plays a pivotal role in mediating platelet aggregation in hemostasis and thrombus formation by inactivating the Src family kinases.[54,55] Recently, it is reported that human patients with *PTPRJ* mutations presented as nonsyndromic thrombocytopenia with the following characteristics: spontaneous bleeding, an increased proportion of small platelets, MK maturation deficiency, and impaired platelet responses to the GPVI agonists collagen and convulxin.[56] To confirm that the *PTPRJ* gene is the IT causative gene, the authors generated a *ptprja* zebrafish mutant by CRISPR/Cas9 and found

that the mutant fish showed decreased *cd41*$^{+}$ thrombocytes, which recapitulates the *PTPRJ*-deficient patient IT syndromes.[56] Consistently, the *Ptprj* mutant mice showed bleeding tendency and were unable to form arterial thrombosis, suggesting that *PTPRJ* is a positive regulator of platelet activation and thrombosis.[57] These findings advance our understanding on the PTPRJ roles in platelet development and functions as well as in pathogenesis.

3.1.4. Nuclear factor erythroid 2 (NFE2) causes IT in animal models. NFE2 is a member of the basic zipper (bZIP) and CAP'n'Collar (CNC) superfamily that regulates gene transcription.[58] *NFE2* has been found to be associated with myeloproliferative disorders, polycythemia vera, and myelofibrosis.[59] *NFE2* gene is expressed in hematopoietic progenitor cells, myeloid, erythroid, and MK lineages[60,61] and regulates proplatelet formation by promoting the MK maturation in mice.[62,63] The *Nfe2* mutant mice showed an absence of circulating platelets, which led to the death of the mice due to extensive hemorrhage.[64] In zebrafish *nfe2* mutant, blood-circulating thrombocytes were severely reduced.[65] The *nfe2* mutant thrombocytes displayed functional perturbation because of fibrinogen-dependent spreading reduction.[65] Although NFE2 mutations have not been revealed in platelet-related disorders, these findings in zebrafish and mice will provide a reference for the discovery of new genes for platelet-related diseases in humans.

3.1.5. Thrombotic thrombocytopenic purpura (TTP) and ADAMTS13. TTP is a life-threatening rare disorder characterized by severe thrombocytopenia, profound hemolytic anemia, abundant schistocytes, neurological deficits, renal injury, and fever.[66] The disorder is mainly caused by acquired autoimmune mechanism but rarely inherited via mutations of the *ADAMTS13* gene,[67] which is required for cleavage of ultralarge vWF multimers. Severe ADAMTS13 deficiency in patients promotes the accumulation of platelet-hyperadhesive ultralarge vWF multimers and the formation of occlusive thrombi in small arterioles and capillaries.[66] However, *Adamts13*$^{-/-}$ mice do not spontaneously develop TTP unless induced by shigatoxin-2 or recombinant human vWF,[68,69] suggesting that additional factors may be necessary for triggering TTP. Recently, Zheng et al created zebrafish *adamts13* mutants by using CRISPR/Cas9 and found that the mutant develops a spontaneous nonfatal TTP phenotype,[70] similar to that in patients with hereditary TTP.[67] Moreover, when induced by histone, *adamts13*$^{-/-}$ zebrafish showed more severe TTP and increased mortality rate. The authors also generated *vwf* mutant and introduced to *adamts13*$^{-/-}$, and found that TTP in *adamts13*$^{-/-}$ zebrafish was restored by genetically deleting *vwf*. Thus, these findings not only provide a zebrafish *adamts13*$^{-/-}$ TTP-like model but also support the crucial physiological function of zebrafish *adamts13* and *vwf* interaction in mediating platelet adhesion/aggregation and thrombus formation.

3.2. Essential thrombocythemia (ET)

ET is a kind of Philadelphia chromosome-negative chronic myeloproliferative disorder, with high incidence of thrombosis and hemorrhage.[71] Generally, thrombocytosis and platelet dysfunction are responsible for the thrombo-hemorrhagic phenomena in ET.[71] It has been reported that several genes are associated with ET, such as JAK2, *CALR*, and *MPL*.

3.2.1. ET and JAK2. JAK/ STAT pathways are important for mediating the cytokine receptor function. JAK2 that belongs to the JAK family plays an important role in hematopoiesis regulation.[72] A gain-of-function V617F mutation in the JH2 domain of JAK2 (JAK2^{V617F}) was identified in myeloproliferative disease (MPD)[73] and ET patients.[74] In zebrafish, overexpressing jak2a^{V581F} (an ortholog of human JAK2^{V617F} mutation) could significantly increase Stat5 phosphorylation and induce a significant increase in erythropoiesis.[75] Overexpressing *jak2a*^{V581F} increased the *cd41:eGFP*^{high} thrombocytes and the expression of thrombocytic markers, resembling the ET phenotype.[17] *Jak2*$^{V617F/WT}$ mice showed an early thrombocytosis because of the expression of *Jak2*^{V617F} in the MKs.[76] Yet, inheritable *jak2a*^{V581F} zebrafish lines are still needed for stably modeling human ET pathogenesis and for drug development.

3.2.2. ET and CALR. CALR protein is a kind of calcium channel binding molecular chaperone, which plays a variety of functions in the body, such as participating in immune response and regulating cell proliferation.[77–79] CALR mutations are associated with about 30% of JAK2 and MPL-unmutated ET and primary myelofibrosis (PMF) patients.[80] In zebrafish, overexpressing human *CALR* mutant mRNA (*CALR*-del52, the most prevalent types of *CALR* mutation in myeloproliferative neoplasms) caused an increase of the hematopoietic stem/progenitor cells followed by thrombocytosis, together with the Jak-Stat signaling activation.[81] Morpholino knockdown of *mpl* could significantly attenuate the effects of mutant CALR, showing the effect of mutant CALR was *mpl* dependent.[81] These findings not only demonstrated CALR roles in hematopoiesis but also indicated that CALR-related signaling for hematological progress are conserved between zebrafish and human.

3.3. Platelet function-related disorders

3.3.1. Gray platelet syndrome (GPS) and NBEAL2. GPS is a predominantly recessive platelet disorder characterized by mild thrombocytopenia with large platelets, α-granule deficiency, and hemorrhages.[82] *NBEAL2* (neurobeachin-like 2), which is highly expressed in MKs and granulocytes and is upregulated during the generation of MKs, plays an important role in MK α-granule development.[83] Researchers have utilized genomic DNA sequencing to identify the *NBEAL2* as the causative gene for GPS.[84,85] Albers et al confirmed that knockdown of *nbeal2* in zebrafish led to a complete thrombocyte abrogation and spontaneous bleeding,[83] resembling mammalian GPS symptoms.[86]

3.4. Understanding thrombocyte regulation in zebrafish

3.4.1. GPR34. GPR34 is an orphan G protein-coupled receptor, and it belongs to the $P2Y_{12}$-like receptor group within the family of rhodopsin-like receptors. The $P2Y_{12}$-like receptor group comprises the ADP receptors ($P2Y_{12}$, $P2Y_{13}$) and the orphan receptors (GPR34, GPR87, GPR82).[87] The cAMP is a platelet activation inhibitor and is produced under the regulation of $P2Y_{12}$-like receptor pathway through reducing adenylyl cyclase activity.[88,89] Zebrafish *gpr34l* is an ortholog of human *GPR34*, and its mutation elevated the cAMP production, prolonged time to occlusion in the laser injury-induced thrombosis formation assay.[90] The thrombocyte aggregation deficiency in the *gpr34l* mutant supports the *gpr34l* roles in regulating thrombocyte function via cAMP. However, whether human *GPR34* mutations link to platelet-related diseases remains to be explored in the future.

4. CONCLUSION AND SPECULATION

The zebrafish has become a model system with a number of unique features that make it a powerful tool for studying

thrombocyte development and disorders during the past decades. Taking advantage of zebrafish thrombocyte-specific markers and lines, the zebrafish is compatible with both traditional and advanced molecular, cellular, and genetic approaches to study thrombocyte biology and pathology in an intact organism. More and more thrombocyte disorders, including CAMT, IT, and ET, and several platelet functional disorders have been resembled successfully in zebrafish, and these zebrafish lines serve as valuable tools for verification of human ortholog gene functions in hematopoiesis and pathogenesis, evaluation of drug responses and risks, and screening of novel genetic and chemical modifiers in thrombocytopoiesis. Despite these progresses, still many clinically common diseases, such as immune thrombocytopenia, need to be modeled in zebrafish for drug discovery. In summary, the zebrafish provides a powerful model system to study hematopoietic and hemostatic disorders, and these zebrafish models will greatly facilitate the discovery of new molecular processes and novel chemical compounds for thrombocytopoiesis and thrombocyte-related disorders in the future.

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