Loss of \textit{Tet2} affects platelet function but not coagulation in mice

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\textbf{Abstract}

Ten-eleven translocation 2 (TET2) functions as a methylcytosine dioxygenase that catalyzes the iterative oxidation of 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxy-cytosine. TET2 has been shown to be crucial for the maintenance and differentiation of hematopoietic stem cells, and its deletion and/or mutations result in the expansion of HSPCs, and leads to hematological malignancies. TET2 mutations were found in a variety of hematological disorders such as CMML (60%), MDS (30%), MPN (13%) and AML (20%). Interestingly, it was shown that CMML patients with TET2 mutation exhibited fewer platelets than CMML patients without TET2 mutation. However, the role and function of TET2 in platelet hemostasis and thrombogenesis is not well defined. Here in this study, using a genetically engineered Tet2 deletion mouse model, we found that the absence of TET2 caused a decrease in the proportion of MEP cells and hyperploid megakaryocytes. Additionally, Tet2-deficient mice displayed impaired platelet activation and aggregation under stimulation of ADP and low concentrations of thrombin, although the modestly compromised platelet function and MEP differentiation in Tet2-deficient mice could be compensated without affecting blood coagulation function. Our study indicate that Tet2 deficiency leads to mild impairment of platelet function and thrombopoiesis in mice.

\textbf{Keywords:} Megakaryocytes, Mouse, Mutation, Platelet, Tet2, Thrombosis

1. INTRODUCTION

Ten-eleven translocation 2 (TET2) is one of the three proteins of the TET family, which contains three evolutionarily conserved dioxygenases including TET1, TET2 and TET3.\(^1\) The catalytic activity of TET dioxygenases requires Fe(II) and \(\alpha\)-ketoglutarate (\(\alpha\)-KG).\(^2\) In recent years, extensive studies of TET2 revealed that its function is not limited to oxidize 5-methyl-cytosine (5mC) to 5-hydroxymethyl-cytosine (5hmC) and promote DNA demethylation on DNA level.\(^3\) Additionally, the TET2–PSPC1 complex could recruit HDAC1/2 to deacetylate histones, thereby inhibiting gene transcription.\(^4\) At the post-transcription level, TET2 could also cause the instability and eventually degrading of targeted RNA by catalyzing RNA 5hmC modification.\(^5\) As an epigenetic regulator, TET2 participates in the regulation of many important biological activities, including early embryonic development and differentiation of hematopoietic stem cells.\(^6\) TET2 mutations were observed in a wide range of hematological tumors with high frequency, such as chronic myelomonocytic leukemia (CMML), myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), and acute myeloid leukemia (AML).\(^7\) Tet2 knockout mouse models have been developed and used in studies. Loss of Tet2 leads to the development of various hematological tumors in mice.\(^8\) The phenotypes of some diseased mice were comparable to those patients with myeloid neoplasm, such as hepatosplenomegaly and myeloproliferation.\(^9\) However, the underlying mechanism is still unclear and needs further studies.

With the extensive application of sequencing technologies in diagnosis, a variety of molecular markers of hematological diseases have been recognized, such as of JAK2 mutations in MPN and CSF3R mutations in chronic neutrophil leukemia (CNL).\(^10\) As a type of chronic myeloid leukemia, CMML is characterized by persistent (>3 months) peripheral blood (PB) monocytosis (>1 x 10\(^9\)l), which accompanied by bone marrow (BM) dysplasia.\(^11\) A number of gene mutations have been identified in CMML, including TET2 (60%), ASXL1 (40%) and...
IDH2 (5%–10%) that are involved in epigenetic regulation. \(^{18,19}\) Notably, there are no unique markers of chromosomal translocation in CMML, and the diagnosis of the disease is mainly based on clinical characteristics. TET2 deletion has been shown to cause excessive proliferation and abnormal differentiation of erythroid progenitor cells. \(^{20,21}\) Intriguingly, Tet2 deficiency facilitated NLPR3-mediated IL-1β production in macrophages, thereby accelerating atherosclerosis development in mice. \(^{22}\) Clinical data have shown that CMML patients with TET2 mutations have fewer platelets than that of wild-type TET2. \(^{23,24}\) These observations suggested that Tet2 might play a role in platelet function and/or coagulation. However, the role of TET2 mutations in hemostasis and thrombosis is still yet to be determined.

Here, using a Tet2-deficient mouse model, we performed a series of experiments to examine the role of Tet2 in megakaryocytes, platelets and blood coagulation. We found that Tet2 deficiency leads to mild impairment of platelet function and thrombopoiesis in mice.

2. RESULTS

2.1. Impaired functions of platelets in Tet2-deficient mice

Platelet activation and blood coagulation are complementary in hemostasis and thrombosis. \(^{25}\) The specific factors released by activated platelets could effectively promote the activation of coagulation factors such as V, VIII and XI, that lead to the activation of prothrombin. \(^{26}\) In turn, thrombin derived from prothrombin can accelerate the activation of platelets. Such a positive feedback process continues until thrombi have been formed by aggregated platelets and activated fibrin. Therefore, we first examined the number and function of platelets in Tet2 knockout mice. We performed the whole blood routine on Tet2-deficient and wild-type mice of 12–16 weeks age. Except for a slight increase in platelet distribution width (PDW), no significant differences were observed in the counts of white blood cells (WBCs), red blood cells (RBCs), platelets (PLTs) and mean platelet volume (MPV) between the two groups (Fig. 1A). To further examine the platelet activation function, various concentrations of strong and weak agonists were utilized to further examine the platelet activation function, various concentrations of strong and weak agonists were utilized to determine the differentiation ability of MEPs, the MegaCult-C system was used to culture the colony of megakaryocyte (CFU-Mk) from isolated whole bone marrow (WBMs) to megakaryocyte-erythroid progenitor cells (MEPs) from Tet2-deficient mice at 8 weeks of age were modestly decreased than that in wild-type mice (Fig. 2B,C), whereas the proportions of common myeloid progenitor cells (CMPs) and granulocyte macrophage progenitor cells (GMPs) had no significant differences between the two groups. To determine the differentiation ability of MEPs, the MegaCult-C system was used to culture the colony of megakaryocyte (CFU-Mk) from isolated whole bone marrow (WBMs) to megakaryocytes of mice (Fig. 2D). Only colonies with positive acetylatedinoliniodide staining cells more than or equal to three were counted as CFU-Mk. We found that the number of CFU-Mk of Tet2-deficient mice were fewer than the wild-type group (Fig. 2E). The production of platelets depends on the number and size of MK, and the size is often determined by its own DNA ploidy. \(^{28,29}\) Furthermore, hypoploid megakaryocytes have been previous indicated to produce platelets more efficiently and effectively. \(^{30}\) We thus further analyzed the ploidyization in megakaryocytes by analyzing the DNA contents of megakaryocyte with Hoechst 33342 staining via flow cytometry. We found a mild decrease in the proportion of high ploidy (>32N) megakaryocytes accompanied with an increase in the proportions of low and medium ploidy (2N, 4N, 8N, 16N) megakaryocytes in Tet2-deficient mice when compared with wild-type mice (Fig. 2F, H). Simultaneously, a slight increase in the proportion of CD41+ cells was observed in Tet2-deficient mice (Fig. 2G). The result suggested that Tet2 deficiency leads to mild defective megakaryocytopoiesis.

2.2. Tet2-deficient mice have reduced proportion of MEP and hyperploid megakaryocytes

The attenuated ability of the aggregation and activation of platelets promoted us to further examine the precursor cells of platelets. Since platelets are derived from megakaryocytes, we wondered whether the absence of Tet2 in mouse had a role in megakaryocytic lineage differentiation and/or maturation. To address this, we firstly examined the proportions of hematopoietic stem and progenitor cells (HSPC) in the bone marrow (BM) from Tet2-deficient and wild-type mice (Fig. 2A). Consistent with previous studies, \(^{13,27}\) the percentages of Lin–Sca–c–Kit+ cells (LKS–) and megakaryocyte-erythroid progenitor cells (MEPs) from Tet2-deficient mice at 8 weeks of age were modestly decreased than that in wild-type mice (Fig. 2B,C), whereas the proportions of common myeloid progenitor cells (CMPs) and granulocyte macrophage progenitor cells (GMPs) had no significant differences between the two groups. To determine the differentiation ability of MEPs, the MegaCult-C system was used to culture the colony of megakaryocyte (CFU-Mk) from isolated whole bone marrow (WBMs) to megakaryocytes of mice (Fig. 2D). Only colonies with positive acetylatedinoliniodide staining cells more than or equal to three were counted as CFU-Mk. We found that the number of CFU-Mk of Tet2-deficient mice were fewer than the wild-type group (Fig. 2E). The production of platelets depends on the number and size of MK, and the size is often determined by its own DNA ploidy. \(^{28,29}\) Furthermore, hypoploid megakaryocytes have been previous indicated to produce platelets more efficiently and effectively. \(^{30}\) We thus further analyzed the ploidyization in megakaryocytes by analyzing the DNA contents of megakaryocyte with Hoechst 33342 staining via flow cytometry. We found a mild decrease in the proportion of high ploidy (>32N) megakaryocytes accompanied with an increase in the proportions of low and medium ploidy (2N, 4N, 8N, 16N) megakaryocytes in Tet2-deficient mice when compared with wild-type mice (Fig. 2F, H). Simultaneously, a slight increase in the proportion of CD41+ cells was observed in Tet2-deficient mice (Fig. 2G). The result suggested that Tet2 deficiency leads to mild defective megakaryocytopoiesis.

2.3. Tet2 deletion did not affect the coagulation function of mice

The coagulation process is mainly divided into three parts, including the production of prothrombin stimuli, the activation of thrombin and the generation of fibrin. \(^{31}\) The first stage consists of endogenous coagulation pathway and exogenous coagulation pathway in accordance with the source of coagulation factors (Fig. S2A, http://links.lww.com/BS/A24). In order to further explore whether the dysfunction of megakaryocytes and platelets caused by the absence of Tet2 may have an effect on the functions of the coagulation system, we measured multiple parameters of the coagulation cascade in Tet2-deficient and wild-type mice, such as coagulation factors, fibrin level, activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT). Nevertheless, none of these coagulation-related indicators appeared abnormal in Tet2 knockout mice when compared with those of wild-type mice (Fig. 3A–C). To
assess the coagulation function of Tet2-deficient mice in vivo, we also performed tail bleeding assay by observing the bleeding time (Fig. S2B, http://links.lww.com/BS/A24), and we found there was no significant difference in their bleeding time of Tet2-deficient mice and wild-type mice indicating that Tet2 deletion does not affect coagulation function (Fig. 3D).

3. DISCUSSION

TET2 mutations have been frequently found in various hematological malignancies, including in CMML. Interestingly, CMML patients with TET2 mutations show lower platelet counts than patients bearing wild-type TET2. The underlying role and regulatory mechanism of TET2 in platelet hemostasis and thrombosis, we investigated the function of platelet and coagulation system as well as megakaryocyte lineage differentiation in Tet2-deficient mice (Fig. 3E). We observed that platelet aggregation and activation under stimulation of ADP or low doses of thrombin in Tet2-deficient mice were modestly impaired, suggesting that Tet2 is involved in platelet function and blood hemostasis. However, under the stimulation of high concentration of thrombin (0.5 U/ml), the level of P-selectin exposure on the platelets of Tet2-deficient mice was increased. Mouse platelets express PAR3 and PAR4, but not PAR1, which is a key thrombin receptor on human platelets. Notably, the PAR3 depletion only inhibits the activation of mouse platelets at low levels of thrombin but could be overcome by high concentrations of thrombin. Other studies have shown that the signaling pathways that mediate by low or high concentrations of thrombin are different. Thus, our observation that Tet2-deficient platelets...
Figure 2. Tet2-deficient mice have reduced proportion of MEP and hyperploid megakaryocytes. (A) Representative graphs of flow cytometric analysis for progenitor cells in BM of Tet2 KO and WT mice. (B and C) All the numbers shown represented the percentages of different cell populations (Lin–Sca-1–c-Kit+ in Lin–c-Kit+ cell population; CMP, GMP and MEP in Lin–Sca-1–c-Kit+ cell population; n = 4). Quantification of frequencies LKS – (B), as well as CMP, GMP and MEP (C). (D) Photographs of representative megakaryocyte colonies with acetylthiocholiniodide staining from WBM cells of Tet2 KO and WT mice. The WBM cells were cultured in MegaCultTM-C collagen and lipid-containing medium for 6 days, and the number of colonies was counted after staining. The brown-black cells indicated by the red arrow are megakaryocytes. CFU-Mk: ≥3 megakaryocytes; CFU-Mk: 1 or 2 megakaryocytes; CFU-non-Mk: 0 megakaryocytes. Scale bar: 100 μm. (E) Quantification of CFU-Mk colonies in panel D (n = 3). (F) Representative flow data for DNA content (2N, 4N, 8N, >32N) in megakaryocytes from BM cells of Tet2 KO and WT mice. (G and H). The percentages of CD41+ cells (G) and megakaryocytes with different DNA contents (H) from Tet2 KO and WT mice were shown (n = 3). CMP, common myeloid progenitor, Lin–Sca-1–c-Kit+CD16/32–CD34–; GMP, granulocyte-monocyte progenitor, Lin–Sca-1–c-Kit+CD16/32–CD34–; MEP, megakaryocyte-erythrocyte progenitor, Lin–Sca-1–c-Kit+CD16/32–CD34–. The data were shown as means ± SEM. Statistical analysis performed by multiple Student’s t test. P value: *P < .05; ns, not significant.
responded differently to the low or high doses of thrombin is possible.

Studies have shown that aged mice with Tet2 deletion could develop CMML-like diseases. Previously, Moran-Crusio K et al. also found that the loss of Tet2 caused a decrease in the ratio of MEP, which was considered as a concomitant phenomenon of the increase in the proportion of GMP cells. However, other studies demonstrated that Tet2 deletion causes erythroid dysplasia, suggesting that Tet2 deletion may have a direct deleterious effect on differentiation of MEP. In this study, we also observed the proportion of hyperploid cells was decreased in the total megakaryocytes of Tet2-deficient mice. However, although the platelet count was decreased slightly in Tet2-deficient mice, there was no significant difference between the two groups. We reasoned that the declined proportion of MEPs and high ploidy (>32N) megakaryocytes could reduce the platelet counts, while the elevated proportion of CD41 cells might increase the platelet counts, and thus compensate for the impact of less MEP and high ploidy. In addition to being precursor cells of platelets, megakaryocytes are also the integral components of the niche for hematopoietic stem cells (HSCs), and have been shown to effectively regulate the function of HSCs. However, whether Tet2-deficient megakaryocytes play a role in the differentiation of HSC by affecting their niches needs further investigation.

The hemostasis in the body is the results of the interplay between platelets, the coagulation system, and the fibrinolysis system. We thus tested the relevant indicators of the coagulation system and found that the lack of Tet2 did not have a significant effect on them. In addition, we did not observe a significant difference between two groups in tail bleeding assay. It is possible that the mild impairment of platelet function in Tet2-deficient mice did not compromise the basic function of hemostasis. The similar phenomenon was also observed in the other study. Lucia Stefanini et al found that a talin mutant (W359A) could also attenuate the activation of aIIb3 without pathological bleeding, which seems to be related to the tight regulation of integrin affinity.

In summary, we found that Tet2 deletion in mice causes a moderate reduction in platelet function which leads to a less extent in platelet activation and aggregation during thrombotic events. This mild attenuation of platelet function may benefit CMML patients bearing TET2 mutations with an improved prognosis. However, further studies are required.

4. MATERIALS AND METHODS

4.1. Mice and PCR genotyping

All mice were housed at State Key Laboratory of Experimental Hematology (SKLEH), Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences. The
experimental procedures were conducted in accordance with the Institutional Animal Care and Use Committee of the Institute of Hematology. Tet2-deficient mice (Stock No: 023359) constructed by the Anjana Rao group were bought from the Jackson Lab and maintained at SKLEH. According to their manuscript, they constructed a targeting vector comprising upstream and downstream homology arms, FRT flanking neomycin resistance cassette and LoxP flanking central region as shown in Figure S1A, http://links.lww.com/BS/A23. Endogenous Tet2 locus was targeted to generate a conditional allele with LoxP sites flanking exon 8 and exon 10. Mice carrying targeted Tet2 alleles were mated to FLP-E-deficient mice to generate F1 with the neomycin resistance cassette removal from the genome. Subsequently, F1 were mated to CMV-CRE deleter mice to generate whole body Tet2 knockout mice. In order to detect the knockdown efficiency of Tet2, DNA from mouse tail tip was extracted for PCR analysis (Fig. S1B, http://links.lww.com/BS/A23). The primer pairs of PCR were as follows: WT forward, AGCTGATGGAAATG-CAAGG; KO forward, GCCACCTTTA-GAAGCTAT TGG; common reverse, TCTCAGACGAAAAAGACTGC. The wild-type allele was 500 bp, while the Tet2-deficient allele with a deleted fragment was only 200 bp.

4.2. Quantitative RT-PCR

We sorted monocytes from 8-week-old mice via flow cytometry for qRT-PCR assay to validate the silence of Tet2 at mRNA level in Tet2-deficient mice (Fig. S1C, http://links.lww.com/BS/A23). Total RNA from monocytes was extracted using Trizol (Invitrogen). Then a reverse transcription system (Takara) was used to reverse-transcribe mRNA to CDNA. The cDNAs of different samples were amplified by QuanStudio 5 Real-Time PCR Instrument with FastStart Universal SYBR Green Master (Roche). The pairs of primer for detecting mRNA expression on Tet2 were as follows: CAAGAAGCTTGCACCCGGATG; GGCGATCTTCGCTGGTC- TCT. All the data were normalized to β-actin expression and represented relatively to expression in WT mice. Relative expression changes were calculated with 2^ΔΔCt method.

4.3. Blood coagulation function tests

The peripheral blood (PB) of 12-week-old mice were centrifuged at 3000 rpm for 10 minutes and the supernatant was transferred to test tube. Four parameters of coagulation system (activated partial thromboplastin time, prothrombin time, thrombin time and fibrinogen) and coagulation factors (II, V, VII, IX, XI, XII) were detected with Sysmex CS5100 system (activated partial thromboplastin time, prothrombin time, thrombin time and fibrinogen) and coagulation factors (II, V, VII, IX, XI, XII) were detected with Sysmex CS5100.

4.4. Tail bleeding time assay

Eight-week-old mice were anesthetized in an induction box containing 5% isoflurane and maintained at 3% isoflurane with mask. The mice were lying on their stomachs naturally with their tails placing horizontally. Next, tails were quickly cut off with a scalpel 5 mm away from the tip of the tails and then immediately immersed in 0.9% saline at 37°C. The bleeding time was expressed as the sum of bleeding within 10 min of observation, including refill. No blood bleeding within 1 min was considered as stopping bleeding, and the time of complete termination of blood flow was recorded.

4.5. Flow cytometry

Single-cell suspensions were obtained from PB and BM, then stained in PBS solution. Antibodies used in this study were as follows: PE-cyanine7 anti-CD66a (25-4317-82, eBioscience), Biotin anti-CD3e (553060, BD Phарmingen), Biotin anti-CD4 (553728, BD Phарmingen), Biotin anti-CD8a (553029, BD Phарmingen), Biotin anti-ly-6G/ly-6C (553124, BD Phарmingen), Biotin anti-Ter119 (553672, BD Phармingen), Biotin anti-B220 (553086, BD Phарmingen), Biotin anti-CD11b (553509, BD Phармingen), APC anti-CD117 (17-1172-82, eBioscience), FITC anti-CD34 (553733, BD Phармingen), APC-cyanine7 anti-ly-6G/ly-6C (A15424, Invitrogen), PE anti-CD16/32 (MFCR04, Invitrogen), PE anti-CD41 (558040, BD Phарmingen), FITC anti-CD41 (553848, BD Phармingen), PE anti-olb63 (M023-2, Emfret), APC anti-CD62P (17-0626-82, eBioscience), Hoechst 33342 (Sigma-Aldrich). Flow cytometric analyses were performed using an LSR II Flow Cytometer (BD Biosciences). Data were analyzed with the FlowJo X 10.0.7r2 software.

4.6. Mk-CFU measurement

WBM cells of mice were collected in a PBS solution, and 3 × 10^5 cells were seeded in a slide according to the instructions of the Megacult C system (Stemcell technologies). After 6 days, the colonies were fixed and dehydrated, then stained with acetylthiocholiniodide and counted. The reagents used in this experiment were as follows: Megacult™ C Collagen and Medium with Lipids (04974, Stemcell technologies), Double Chamber Slide Kit (08963, Stemcell technologies), Acetylthiocholiniodide (A5751, Sigma), Na2HPO4 (97963, Sigma), CuSO4 (209198, Sigma), K3Fe(CN)6 (244023, Sigma), Harris’ hematoyxin solution (HHS16, Sigma), Acetone (179124, Sigma), rh Thrombopoietin (TPO) (300-18, Peprotech), rh IL-6 (200-06, Peprotech), rh IL-11 (220-11, Peprotech), rm IL-3 (213-13, Peprotech).

4.7. MK DNA ploidy analysis

The experiment was conducted according to the previous research. BM cells were stained in a PBS solution containing CD41-PE antibody and incubated on ice for 30 minutes. After incubation, labeled cells were washed in 1 ml PBS by centrifuging at 1500 rpm for 5 minutes. The supernatant was discarded, followed by 1 ml PBS with Hoechst 33342 (final concentration 10 μg/ml) adding to resuspend. Incubate at 37°C protected from light for 1 hour in a water bath. After washing as before, 300 μl PBS was added to resuspend. During FACS analysis, Hoechst 33342 was taken the log value. The proportion of megakaryocytes in WBM cells was less than 0.1%.

4.8. Blood routine tests

Blood was diluted with PBS (PBS with 2% fetal bovine serum and 2 mM EDTA) in 1:4, and the detection of cell blood count (CBC) was performed using Sysmex XT-2000i.

4.9. Platelet activation assay

Blood was collected from ophthalmic venous plexus of anesthetized 12-week-old mice in the presence of 10x citrate-dextrose solution ACD (85 mM sodium citrate dihydrate, 71.4 mM citric acid, 111 mM glucose). Then it was incubated with Hepes-Tyrode’s buffer (pH 7.4, 138 mM NaCl, 2.9 mM KCl, 0.42 mM NaH2PO4·H2O, 12 mM NaHCO3, 2 mM MgCl2·6H2O, 10 mM Hepes, 5.5 mM glucose, and 0.1% bovine serum albumin) containing 1 mM Ca2+, agonists (thrombin or ADP, Chrono-log) and mixed antibodies (FITC anti-CD41, APC anti-CD62P and PE anti-olb63) at room temperature in the dark for 15 minutes. Before using a flow cytometer, appropriate 1% PFA was added to the mixture for fixing cells. Threshold of FSC changed to a minimum value of 200. Reagent formulations were referred to previous research.
4.10. Isolation of washed platelets  
Whole blood was prepared from mice anesthetized with avertin (T48402, Sigma) via the inferior vena cava and collected in 1/9 volume of ACD. All procedures were done at room temperature. PRP was separated from whole blood by centrifuging at 1100 rpm for 10 minutes. Platelets were isolated from PRP by centrifuging at 3500 rpm for 10 min to remove PPP. The pellet was washed with CS buffer twice (pH 7.0, 13 mM sodium citrate dihydrate, 120 mM NaCl, 30 mM glucose) in the presence of 0.1 mM prostaglandin E1 (PGE1). Washed platelets were resuspended in Hapes-Tyrode’s buffer to a final concentration of 2 x 10^8/ml or 3 x 10^7/ml. After recovering at 37°C for 1 hour, washed platelets could be used for subsequent experiments.16,47

4.11. Adhesion and spreading assays of washed platelets  
Poly-d-Lysine/Laminin glass coverslips were coated with 50 μg/ml fibrinogen overnight at 4°C followed by washing with PBS three times. The coverslips were then blocked with 1% BSA in PBS for 1 hour, followed by washing with PBS. The mixture of washed platelets (3 x 10^7/ml) and agonists was transferred to coverslips and incubated at 37°C for 2 hours. The coverslips were washed in PBS pre-warmed to 37°C three times. FITC-488-phalloidin immunofluorescence staining was performed at 37°C overnight after fixation for 20 minutes by 4% paraformaldehyde, permeating for 1 hour by 0.2% Triton X-100 and closing for 30 minutes by 1% BSA in proper order. Coverslips were snapped onto the slides and added antifade mounting medium (with DAPI). All the samples were observed and taken images with UltraView VOX (PerkinElmer) and analyzed with Velocity software.

4.12. Aggregation assay of washed platelets  
For the aggregation assay, the concentrations of washed platelets were adjusted to 2 x 10^7/ml with Hapes-Tyrode’s buffer supplemented with 1mM CaCl2. Aggregations of washed platelets were examined by Chrono-log Model 700 Whole Blood/Optical Lumi-Aggregometers.

4.13. Statistical analysis  
Data were expressed as means ± SEM. All calculations were measured using GraphPad Prism 6.0 software. Experiments were performed in three times and repeated. The Student t test was applied to compare the results. When the P value was less than .05, the result was defined as a significant difference.

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