Loss of *Tet2* affects platelet function but not coagulation in mice

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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-carboxylcytosine. TET2 has been shown to be crucial for the maintenance and differentiation of hematopoietic stem cells, and its deletion and/or mutations results 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.

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 α-ketoglutarate (α-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.[1] Additionally, the TET2–PSPC1 complex could recruit HDAC1/2 to deacetylate histones, thereby inhibiting gene transcription.[3] At the post-transcription level, TET2 could also cause the instability and eventually degrading of targeted RNA by catalyzing RNA 5hmC modification.[4–6] As an epigenetic regulator, TET2 participates in the regulation of many important biological activities, including early embryonic development and differentiation of hematopoietic stem cells.[7–9] *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).[10] *Tet2* knockout mouse models have been developed and used in studies. Loss of *Tet2* leads to the development of various hematological tumors in mice.[11–13] The phenotypes of some diseased mice were comparable to those patients with myeloid neoplasm, such as hepatosplenomegaly and myeloproliferation.[14] 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).[15,16] As a type of chronic myeloid leukemia, CMML is characterized by persistent (>3 months) peripheral blood (PB) monocytosis ($>1 \times 10^9$/l), which accompanied by bone marrow (BM) dysplasia.[17,18] A number of gene mutations have been identified in CMML, including *TET2* (60%), *ASXL1* (40%) and

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Authors' contributions: Y. Chu and W. Yuan conceived the project, supervised the research and revised the manuscript. B. Wang conceived and designed the study, collected and assembled the data, analyzed and interpreted the data, and wrote the manuscript. M. Xia, T. Chen, M. Li and D. Shi helped with the platelet-related experiments. X. Wang, A. Pang and J. Zhou provided study materials and paper discussion.

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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 NLRP3-mediated IL-1b 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 stimulate platelets, respectively. Thrombin was used as a strong agonist at a concentration of 0.02, 0.1, and 0.5 U/ml, while ADP as a weak agonist at a concentration of 20, 100, 500 μM. By measuring the median fluorescence intensity (MFI) of the surface markers CD62P and αIIbβ3 that represented the activation of platelets, we found that the activation of platelets from *Tet2*-deficient mice were weaker in comparison with wild-type mice under the treatment of ADP and low dose of thrombin (0.02 U/ml) (Fig. 1B). Unexpectedly, 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. Platelet aggregation is essential for the entire hemostatic process, and its aggregation ability is viewed as a golden standard in evaluation of platelet function. Thus, we further analyzed the platelet aggregation ability using washed platelets. Different concentrations of thrombin and ADP were used to stimulate the washed platelets, and we observed that *Tet2*-deficient platelets showed diminished aggregation response to ADP or low concentrations of thrombin. The impairment in aggregation could be overcome when stimulated with high concentrations of thrombin (Fig. 1C). We further assessed the adhesion and spreading ability of WT and *Tet2* KO platelets. We found the adhesion and spreading of washed platelets were similar in WT and *Tet2* KO platelets when stimulated with different concentrations of agonists (Fig. 1D,E). Collectively, our study showed that *Tet2* deletion in mice impaired the platelet ability in activation and aggregation under the stimulation of ADP or low doses of thrombin while the blood routine and platelet count showed no changes.

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-1−c-Kit+ cells (LKS−) and megakaryocyte-erythroid progenitor cells (MEPs) from *Tet2*-deficient mice at 8 weeks of age were modestly decreased than those 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 (WBM) cells of mice (Fig. 2D). Only colonies with positive acetylthiocholiniodide 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, hyperploid megakaryocytes have been previous indicated to produce platelets more efficiently and effectively.[30] We thus further analyzed the ploidization 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

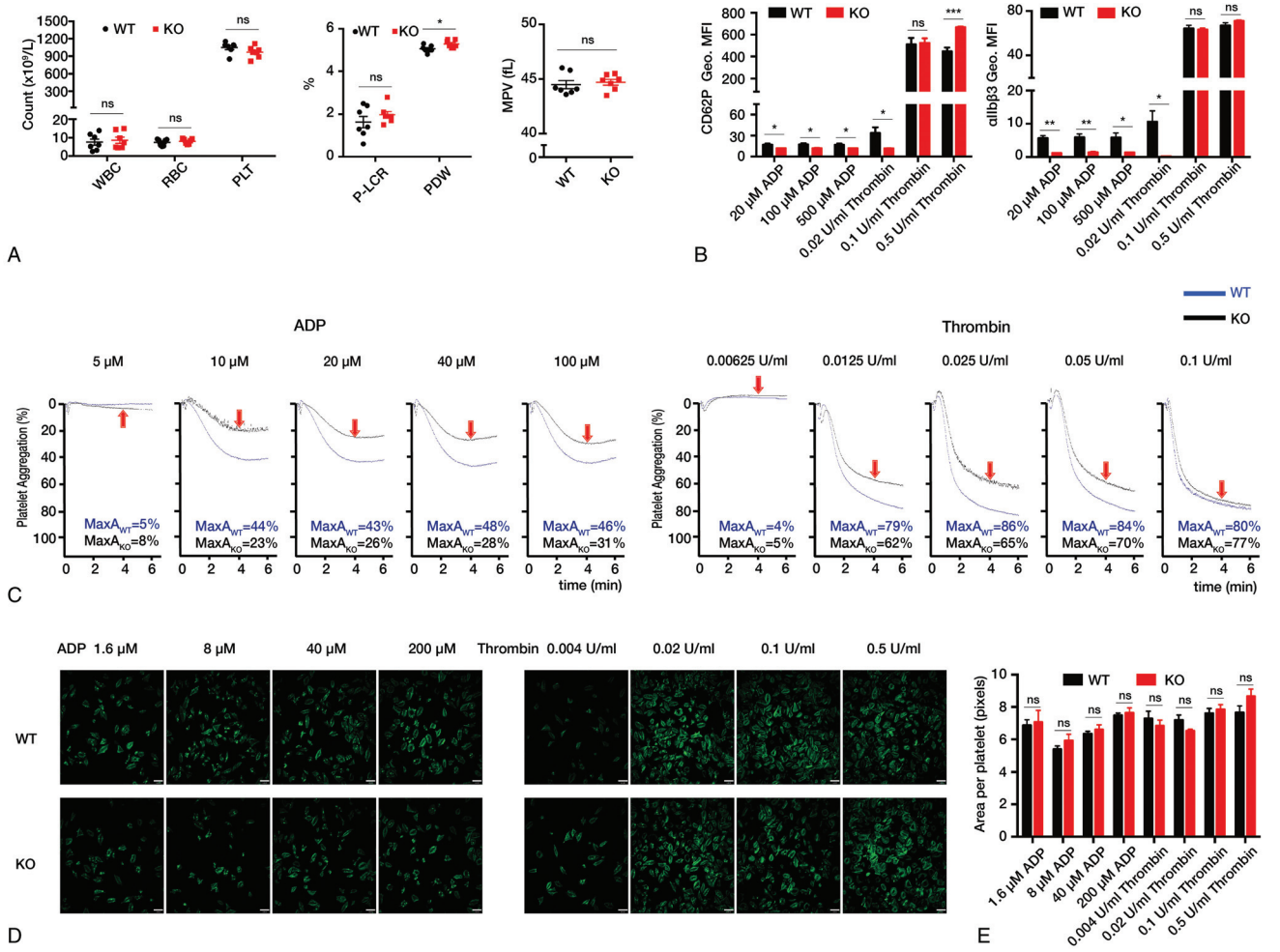

Figure 1. Decreased functions of platelets in *Tet2*-deficient mice. (A) The blood routine results of WT and *Tet2* KO mice showed no significant differences in their WBC, RBC, and PLT counts (left panel), and in P-LCR (middle panel) and MPV (right panel), (*Tet2* KO, n = 7; WT, n = 7). Only PDW (middle panel) was increased moderately. (B) Flow cytometry was used to evaluate PB platelet activation with agonists in *Tet2* KO (n = 4) and WT (n = 5) mice. CD62P (left) and αIIbβ3 (right) were the surface markers used for detection of platelet activation. (C) Aggregation assays of washed platelets (2×10^8/ml) were performed with Chrono-log Model 700 Whole Blood/Optical Lumi-Aggregometers. When stimulated by ADP (right) and medium concentrations of thrombin (left), the aggregation of washed platelets of *Tet2* KO mice was lower than WT mice. Black curve and red arrow represent *Tet2* KO platelets while blue one represents WT platelets. MaxA, maximum aggregation (%). (D and E) Phalloidin-FITC staining of washed platelets (3×10^7/ml) with or without agonists from PB of *Tet2* KO and WT mice. The representative images were displayed (D). Scale bar: 6 μm. Quantification of the area of each platelet (E, n = 3). Data was processing with Volocity software. MPV, mean platelet volume; PB, peripheral blood; PDW, platelet distribution width; P-LCR, platelet-larger cell ratio; PLT, platelet; RBC, red blood cell; WBC, white blood cells. The data were shown as means ± SEM. Statistical analysis performed by unpaired, multiple Student's *t* test. *P* value: $^{*}P < .05$, $^{***}P < .001$; ns, not significant.

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*.[24,32] The underlying role and regulatory mechanism of TET2 in platelet hemostasis and thrombosis were not clear. Considering the vital role of platelet and coagulation in regulating inflammation and thrombosis,[33] 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.[34] Notably, the PAR3 depletion only inhibits the activation of mouse platelets at low levels of thrombin but could be overcame by high concentrations of thrombin.[35] Other studies have shown that the signaling pathways that mediate by low or high concentrations of thrombin are different.[36–38] Thus, our observation that *Tet2*-deficient platelets

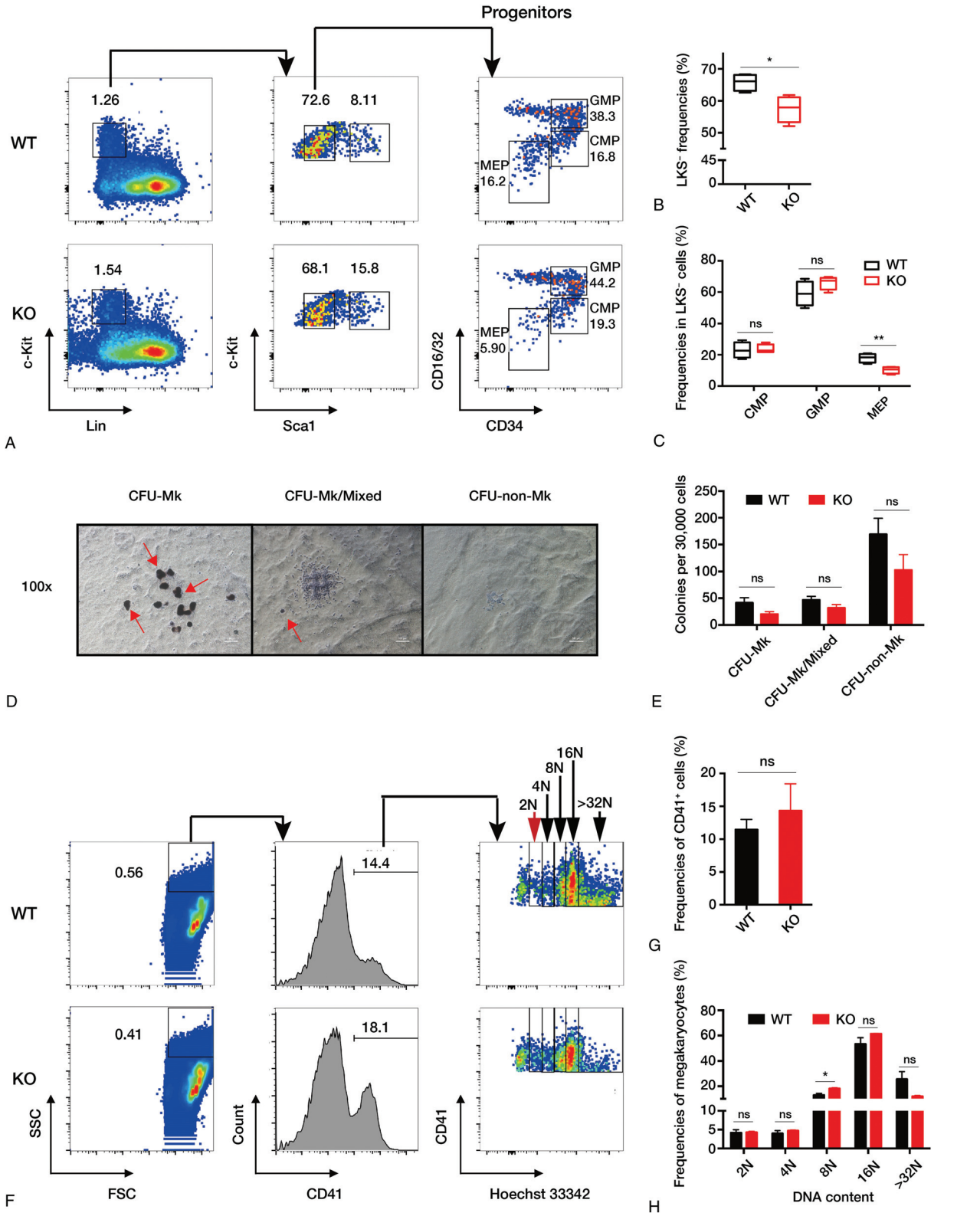

Figure 2. *Tet2*-deficient mice have reduced proportion of MEP and hyperploid megakaryocytes. (A) Representative graphs of flow cytometric analysis for progenitor cells in BM of *Tet2* KO and WT mice. (B and C) All the numbers shown represented the percentages of different cell populations (Lin−Sca-1−c-Kit+ 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, 16N, >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^{low}CD34+; GMP, granulocyte-monocyte progenitor, Lin−Sca-1−c-Kit+CD16/32^{high}CD34+; MEP, megakaryocyte-erythrocyte progenitor, Lin−Sca-1−c-Kit+CD16/$32^{low/neg}$$CD34^{low/neg}$. The data were shown as means ± SEM. Statistical analysis performed by multiple Student's *t* test. *P* value: $^*P < .05$; ns, not significant.

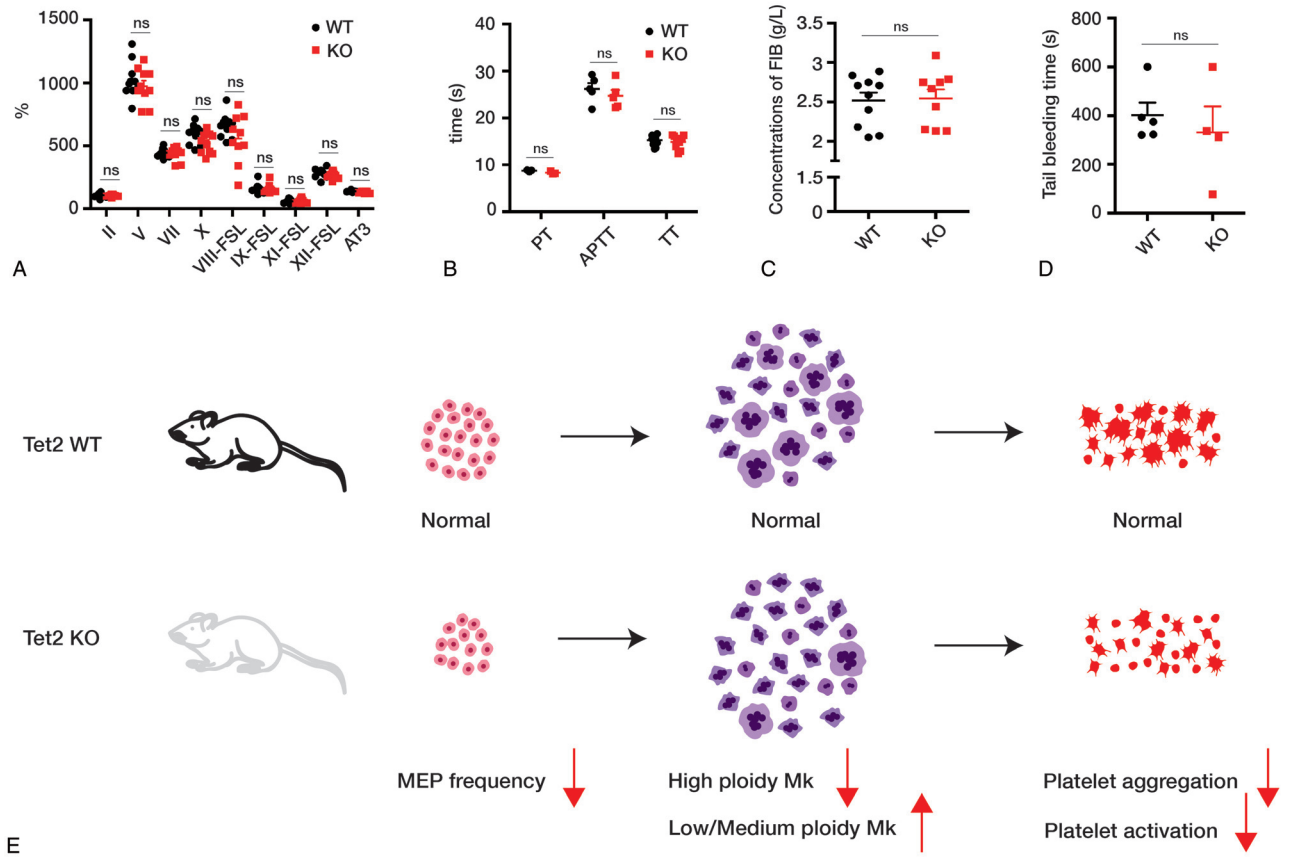

Figure 3. *Tet2* deletion does not change the coagulation function of the *Tet2* KO mice. (A–C) Detection of related indicators of coagulation process. (A) Coagulation factors and AT3 measurement (II, V, VII, X, VIII, IX, XI, XII, n = 10; AT3, n = 7). (B) Measurement of APTT, PT and TT (APTT, n = 5; PT, n = 3; TT, n = 5). (C) FIB content measurement (WT = 10, KO = 9). (D) Tail bleeding time assay by aerosol anesthesia in *Tet2* KO (n = 4) and WT (n = 5) mice. (E) Schematic overview of the role of *Tet2* deficiency on platelet function and thrombopoiesis in mice. APTT, activated partial thromboplastin time; AT3, antithrombin III; FIB, fibrinogen; PT, prothrombin time; TT, thrombin time. The data were shown as means ± SEM. Statistical analysis performed by unpaired, multiple Student's *t* test; 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.[13] Previously, Moran-Crusio K et al. also found that the loss of *Tet2* caused a decrease in the ratio of MEP,[27] 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,[20,21] 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.[39] 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.[40] 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 αIIbβ3 without pathological bleeding, which seems to be related to the tight regulation of integrin affinity.[41]

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.[42] 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 deletor mice to generate whole body *Tet2* knockout mice. In order to detect the knockout 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, AGCTGATGGAAAATG-CAAGC; KO forward, GCCACTTTA-GAAGCCTAT TGGA; common reverse, TCTCAGAGCAAAGAGGACTGC. 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: CAAGAAGCTTGCACCCGATG; GGCATGTTCTGCTGGTC-TCT. All the data were normalized to β-actin expression and represented relatively to expression in WT mice. Relative expression changes were calculated with $2^{-\Delta\Delta Ct}$ 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, X, VIII, IX, XI, XII) were detected with Sysmex CS5100 according to the merchant manual. All reagents of tests were purchased from Siemens.

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 reflow. No blood bleeding within 1 min was considered as stopping bleeding, and the time of complete termination of blood flow was recorded.[43,44]

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-streptavidin (25-4317-82, eBioscience), Biotin anti-CD3e (553060, BD Pharmingen), Biotin anti-CD4 (553728, BD Pharmingen), Biotin anti-CD8a (553029, BD Pharmingen), Biotin anti-Ly-6G/Ly-6C (553124, BD Pharmingen), Biotin anti-Ter119 (553672, BD Pharmingen), Biotin anti-B220 (553086, BD Pharmingen), Biotin anti-CD11b (553309, BD Pharmingen), APC anti-CD117 (17-1172-82, eBioscience), FITC anti-CD34 (553733, BD Pharmingen), APC-cyanine7 anti-Ly-6G/Ly-6C (A15424, Invitrogen), PE anti-CD16/32 (MFCR04, Invitrogen), PE anti-CD41 (558040, BD Pharmingen), FITC anti-CD41 (553848, BD Pharmingen), PE anti-αIIbβ3 (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 3×10^4 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^{TM}-C Collagen and Medium with Lipids (04974, Stemcell technologies), Double Chamber Slide Kit (04963, Stemcell technologies), Acetylthiocholiniodide (A5751, Sigma), Na_2HPO_4 (S9763, Sigma), $CuSO_4$ (209198, Sigma), $K_3Fe(CN)_6$ (244023, Sigma), Harris' hematoxylin 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.[45] 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 PBE (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 $NaH_2PO_4{\cdot}2H_2O$, 12 mM $NaHCO_3$, 2 mM $MgCl_2{\cdot}6H_2O$, 10 mM Hepes, 5.5 mM glucose, and 0.1% bovine serum albumin) containing 1 mM Ca^{2+}, agonists (thrombin or ADP, Chrono-log) and mixed antibodies (FITC anti-CD41, APC anti-CD62P and PE anti-αIIbβ3) 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.[43]

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 CGS buffer twice (pH 7.0, 13 mM sodium citrate dihydrate, 120 mM NaCl, 30 mM glucose) in the presence of 0.1 μM prostaglandin E1 (PGE1). Washed platelets were resuspended in Hepes-Tyrode's buffer to a final concentration of 2×10^8/ml or 3×10^7/ml. After recovering at 37°C for 1 hour, washed platelets could be used for subsequent experiments.[46,47]

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 (3×10^7/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 fixing 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 Volocity software.

4.12. Aggregation assay of washed platelets

For the aggregation assay, the concentrations of washed platelets were adjusted to 2×10^8/ml with Hepes-Tyrode's buffer supplemented with 1 mM $CaCl_2$. 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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