

***Myh9* R702C is associated with erythroid abnormality with splenomegaly in mice**

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ABSTRACT

MYH9 disorders are characterized by giant platelets, thrombocytopenia, and Döhle body-like cytoplasmic inclusion bodies in granulocytes. However, whether these disorders cause any changes in erythroid cells has yet to be determined. This study analyzed the influence of *Myh9* R702C, as one of the most commonly detected *MYH9* disorders, on erythroid cells in a mouse model.

Knock-in mice expressing *Myh9* R702C mutation either systemically or specific to hematological cells (R702C and R702C *vav1* mice, respectively) were used in this study. Both displayed lower hemoglobin and higher erythropoietin levels than wild-type (WT) mice, along with significant splenomegaly. Flow cytometric analysis revealed erythroblasts present at a higher rate than WT mice in the spleen. However, no obvious abnormalities were seen in erythroid differentiation from megakaryocyte/erythroid progenitor to erythrocyte. Cell culture assay by fetal liver and colony assay also showed normal progression of erythroid differentiation from erythroid burst-forming unit to red blood cell.

In conclusion, R702C and R702C *vav1* mice displayed erythroid abnormality with splenomegaly. However, erythroid differentiation showed no obvious abnormality. Further research is required to elucidate the underlying mechanisms.

Keywords: *MYH9* disorders, anemia, mouse model

Abbreviations:

myosin IIA: Non-muscle myosin heavy chain-IIA

EPO: erythropoietin

RBCs: red blood cells

MEP: megakaryocyte/erythroid progenitors

BM: bone marrow

CFU-E: erythroid colony-forming units

BFU-E: erythroid burst-forming units

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Hb: hemoglobin
MCH: mean corpuscular hemoglobin
BW: body weight
Ht: hematocrit
MCV: mean corpuscular volume
MCHC: mean corpuscular hemoglobin concentration
RDW: RBC distribution width
RET: reticulocyte

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INTRODUCTION

MYH9 disorders are autosomal dominant diseases characterized by thrombocytopenia, giant platelets and leukocyte inclusion bodies that are caused by variants in *MYH9*, which encodes non-muscle myosin heavy chain-IIA (myosin IIA).¹⁻³ Myosins are motor proteins that work with actin and constitute a large superfamily of proteins involved in the motor functions of the cell. In humans, three different non-muscle myosin II heavy chains have been identified: IIA, IIB and IIC. Myosin IIA is expressed in hematological cells, kidneys, and the cochlea.

Some groups have investigated the role of myosin IIA in erythroid differentiation. Expression of myosin IIA is known to be maintained during erythroid differentiation, although the expression of non-muscle myosin heavy chain-IIB is decreased toward the late phase of erythroid differentiation.⁴

Keerthivasan et al reported inhibition of myosin II by blebbistatin disturbed erythroid differentiation of primary murine erythroblasts in vitro.⁵ The mechanism was considered to involve the important role of myosin II in contracting the actomyosin ring during cytokinesis,^{6,7} resulting in inhibition of myosin II affecting cytokinesis in erythroid differentiation. However, Keerthivasan et al also reported that this inhibition was not found to disturb enucleation.⁵ Ubukawa et al reported in a study using human erythroblasts that inhibition of myosin IIA did not affect enucleation, while non-muscle myosin heavy chain-IIB was involved in enucleation.⁸ These reports indicated that myosin IIA plays some role in the cytokinesis of erythroid differentiation, but not in enucleation.

Smith et al investigated the role of myosin IIA in the membrane of red blood cells (RBCs) and reported that myosin IIA interacts with the spectrin-actin membrane skeleton and control membrane curvature and deformity in RBCs,⁹ causing abnormal red blood cell morphology, and clinically resulting in lower mean corpuscular hemoglobin concentration (MCHC) and a higher RBC distribution width (RDW) in patients with *MYH9* disorders compared with healthy controls.¹⁰

The *MYH9* R702C variant, located in the head domain, is a commonly detected variant in patients with *MYH9* disorders. Patients with this variant are known to show a more severe clinical phenotype than patients with other variants.¹¹⁻¹³ Our group previously constructed a *Myh9* R702C knock-in mouse model that reproduced *MYH9* disorders very closely.¹⁴ The present study estimated the clinical phenotypes of erythrocyte and erythroid differentiation in *Myh9* disorders using a mouse model.

MATERIALS AND METHODS

Mice

Myh9 R702C heterozygous knock-in mice (R702C mice) that systemically expressed *Myh9* R702C were previously generated by Cre loxP system on a C57BL/6J (The Jackson Laboratory, #000664) background in our laboratory.¹⁴ To create hematopoietic system-specific *Myh9* R702C heterozygous knock-in mice (R702C *vav1* mice), *Myh9* R702C Neo mice (R702C Neo mice) were crossed with *vav1*-Cre transgenic mice (The Jackson Laboratory, #008610). C57BL/6J mice were employed as wild-type (WT) mice. Male mice (20±5 weeks old) were used in all experiments in this study. All research procedures involving animals were performed in accordance with the regulations on animal experiments at Nagoya University. The protocol was approved by the committee on the Ethics of Animal Experiments at Nagoya University Graduate School of Medicine (permit number 20267).

Hematological analysis

Blood was obtained from the aorta of anesthetized mice, then anti-coagulated by ethylenediamine tetra-acetic acid (EDTA). Complete blood cell counts were determined using an automated blood cell analyzer (SE9000; Sysmex, Kobe, Japan).

Erythropoietin (EPO) antigen measurement

Plasma EPO concentrations were measured using Quantikine® ELISA Mouse Erythropoietin Immunoassay (R&D Systems, Minneapolis, MN), in accordance with the instructions from the manufacturer.

Cell preparation for flow cytometric analysis

Bone marrow (BM) cells were freshly isolated by flushing the femora of mice with cold phosphate-buffered saline (PBS) and strained through a 40-µm nylon strainer. Splenic cells were prepared by mincing the organ and then passing the cells through a 40-µm nylon mesh with PBS. The resulting cells were collected into the buffer (PBS with 0.5% bovine serum albumin (BSA) and 2 mM EDTA) and subjected to flow cytometric analysis.

Quantitative analysis of murine erythroid differentiation in BM and spleen

Analysis was performed according to the method reported by Liu et al.¹⁵ BM and splenic cells were centrifuged at 300×g for 10 min at 4°C. Cells were resuspended into the buffer, with 90 µL of buffer per 10⁷ cells, to which 10 µL of mouse CD45 MicroBeads (Miltenyi Biotec, Auburn, CA) was added and then incubated for 30 min at 4–8°C. After adding 1 mL of buffer per 10⁷ cells, CD45-negative cells were separated by magnetic separation with LD columns according to the instructions from the manufacturer. CD45-negative cells were stained with PE-conjugated rat anti-mouse Ter119, APC-Cy7-conjugated rat anti-mouse CD11b and GR1, and APC-conjugated rat anti-mouse CD44 (BioLegend, San Diego, CA). GR1-CD11b-Ter119+ cells were analyzed as Ter119 versus CD44 and CD44 versus FSC.

Erythroid differentiation analysis from fetal liver

In vitro erythroid differentiation analysis was performed according to the previously reported method.¹⁶ In brief, fetal liver cells were collected from fetuses at 14.5 gestational days for WT and R702C+/- mice. Ter119-negative cells were then purified using Ter119-conjugated magnetic beads and LD columns according to the instructions from the manufacturer (Miltenyi Biotec, Auburn, CA). Purified cells were seeded and cultured with IMDM (Life Technologies, Grand

Island, NY) containing 15% fetal bovine serum, 1% BSA (Sigma, St Louis, MO), 200 µg/mL holo-transferrin (Life Technologies), 10 µg/mL recombinant human insulin (Life Technologies), 10⁻⁴ M 2-mercaptoethanol (Life Technologies), and 2 U/mL recombinant mouse EPO (R&D Systems, Mckinley, MN) in fibronectin-coated wells (BD Discovery Labware, Bedford, MA). On the second day, EPO was removed. Flow cytometric analysis was carried out on day 0, day 1 and day 2, using PE-conjugated rat anti-mouse Ter119 antibody and FITC-conjugated rat anti-mouse CD71 (BioLegend) antibody.

Analysis of megakaryocyte/erythroid progenitors (MEPs), erythroid colony-forming units (CFU-E) and erythroid burst-forming units (BFU-E) in BM and splenic cells

BM and splenic cells were prepared for flow cytometric analysis. Lineage-negative cells were isolated from BM or splenic cells. The c-kit^{mediate-high}Sca-1^{low}CD41^{low}FcγRII/III^{low} cells were then identified by CD150 and Endoglin (BioLegend), and divided by the four resins.¹⁷

Colony assays

CFU-E and BFU-E were analyzed using BM cells (5×10⁵ cells/dish), according to the manufacturer's protocol (StemCell Technologies, Vancouver, Canada). Methocult M3334 (StemCell Technologies) was used for CFU-E and BFU-E colonies determined after 2 days and 9 days of culture, respectively.

RESULTS

Generation of R702C vav1 mice

R702C mice were initially employed in the study. However, R702C mice showed a breeding problem, due to a short lifespan (around 20 weeks) and low birth rate. What was more, these mice developed renal disorder from 5 weeks old. This made it difficult to exclude the possibility of renal anemia and led us to generate R702C vav1 mice expressing *Myh9* R702C specific to hematopoietic cells. Confirmation of knock-in was performed using DNA from tail tissue and blood and revealed that the Neo cassette was removed from blood samples, but remained in tail tissues (Supplemental Fig. 1). R702C vav1 mice displayed the same hematological features as R702C mice, with thrombocytopenia, giant platelets and inclusion bodies in granulocytes, but did not show albuminuria on urine test papers (data not shown). These results indicated that *Myh9* R702C was expressed correctly, specific to hematopoietic cells in R702C vav1 mice.

R702C and R702C vav1 mice resulted in erythroid abnormality with splenomegaly

Complete blood count data are shown in Table 1. In comparison with WT mice, R702C vav1 mice displayed significant differences in hemoglobin (Hb), MCHC, platelet count, EPO and weight ratio of spleen/body weight (BW). R702C mice displayed significant differences in Hb, hematocrit (Ht), mean corpuscular volume (MCV), MCHC, RDW-standard deviation (SD) and platelet count. The R702C variant caused hematopoietic abnormalities not only in platelets, but also in erythroid cells. Although EPO was elevated in R702C vav1 mice, reticulocytes (RET) were not, indicating that erythropoiesis did not respond to stimulation by EPO.

R702C mice displayed decreased erythroblast ratio in BM

To assess erythropoiesis in BM *in vivo*, the assay reported by Liu et al was employed (Fig. 1).¹⁵ This analysis enables erythroblasts to be distinguished from erythrocytes (Supplemental Fig. 2). BM cells depleted by CD45-positive cells were subjected to flow cytometric analysis

Table 1 Myh9 R702C mutation causes low Hb with elevated EPO level and splenomegaly

	WT (n=22)	R702C vav1 (n=12)	R702C (n=15)
WBC (μL)	7965 \pm 2370	8447 \pm 4635	5553 \pm 927
RBC ($\times 10^4/\mu\text{L}$)	1021 \pm 45	970 \pm 114	990 \pm 55
Hb (g/dL)	14.7 \pm 0.7	13.6 \pm 1.5*	13.8 \pm 0.8*
Ht (%)	53.6 \pm 2.9	50.3 \pm 6.0	48.7 \pm 3.3*
MCV (fL)	52.6 \pm 1.2	51.8 \pm 1.3	49.1 \pm 0.5*
MCH (pg)	14.4 \pm 0.2	14.0 \pm 0.4*	13.8 \pm 0.1*
MCHC (%)	27.3 \pm 0.6	27.0 \pm 0.6	28.0 \pm 0.3
RDW-SD	24.0 \pm 2.2	25.2 \pm 1.7	27.9 \pm 0.4*
RET (%)	33.3 \pm 4.5	34.0 \pm 6.3	-
Platelet count ($\times 10^3/\mu\text{L}$)	1513 \pm 220	220 \pm 92*	139 \pm 0.8*
Erythropoietin (pg/mL)	64.2 \pm 23.9 (n22)	110.3 \pm 35.8* (n=10)	451.2 \pm 632.7 (n=8)
weight ratio of Spleen/body weight ($\times 10^3$)	2.49 \pm 0.48	3.34 \pm 1.01*	-

*: $p < 0.05$ (In comparison with WT mice)

WBC: white blood cell

RBC: red blood cell

Hb: hemoglobin

Ht: hematocrit

MCV: mean corpuscular volume

MCH: mean corpuscular hemoglobin

MCHC: mean corpuscular hemoglobin concentration

RDW: RBC distribution width

RET: reticulocyte

and identified by a combination of Ter119, FSC and CD44. Ter119-positive cells were analyzed by CD44 and FSC, which revealed that $\text{FSC}^{\text{high}}\text{CD44}^{\text{high}}$ cells were immature cells comprising erythroblasts including proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts and orthochromatic erythroblasts, represented as region I. $\text{FSC}^{\text{low}}\text{CD44}^{\text{low-mediate}}$ cells were mature cells after enucleation consisting of reticulocytes and RBCs, represented by region II. Cells in region I were decreased in R702C mice (2.3%) compared with WT mice (9.6%) (Fig. 1A, 1B), while average erythroblast rates from four serial experiments were 3.6% in R702C mice and 10.7% in WT mice, respectively. These results indicate that the R702C variant caused decreased erythropoiesis in BM.

Numbers of BFU-E and CFU-E were comparable between R702C vav1 and WT mice in colony assays

To identify BFU-E and CFU-E, as progenitor cells of erythroblasts (Supplemental Fig. 2), BM cells were cultured in methylcellulose medium (Methocult M3334; StemCell Technologies). CFU-E were counted on day 2, with counts per dish of 199 \pm 53 in WT and 174 \pm 25 in R702C-vav1 mice. BFU-E were counted on day 9, with counts per dish of 47 \pm 8 in WT and 59 \pm 13 in R702C-vav1 mice (Supplemental Table 1). No significant differences in BFU-E or CFU-E were seen between WT and R702C vav1 mice. This result revealed that production of progenitor cells for erythroblasts was normal in R702C vav1 mice, indicating that erythroid abnormalities developed after the BFU-E or CFU-E stage.

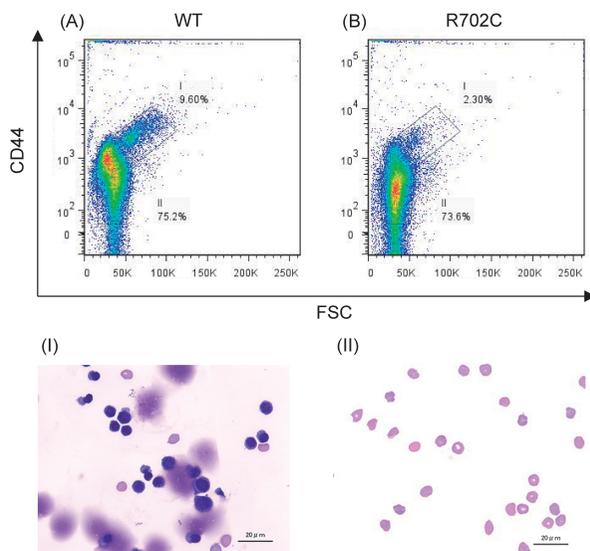


Fig. 1 R702C mice display decreased erythroblasts, but normal erythroid differentiation from proerythroblasts in BM
Fig. 1I-1II: Gate I consists of proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts and orthochromatic erythroblasts, categorized as erythroblasts. These cells are shown as (I). Gate II contains reticulocytes and red blood cells, shown as (II).

Fig. 1A-1B: Erythroblasts are decreased in R702C (2.3%) (B) compared with WT (9.6%) (A). Experiments repeated four times yield average erythroblast rates of 3.6% for R702C mice and 10.7% for WT mice. BM: bone marrow

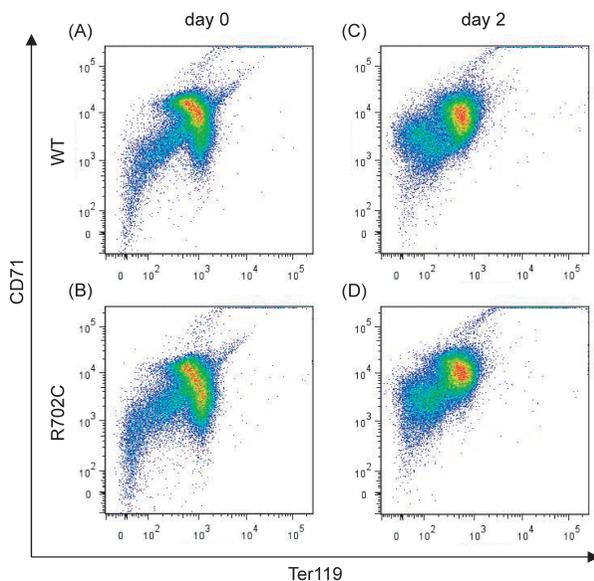


Fig. 2 Erythroid differentiation from BFU-E in fetal liver cell culture

Fig. 2A-2B: Fetal liver cells obtained from fetuses at E14.5 gestational days are subjected to flow cytometric analysis. No significant differences are seen between WT and R702C mice.

Fig. 2C-2D: Ter119-positive cells removed from fetal liver cells and cultured with Epo for two days are then analyzed by flow cytometric analysis on days 1 and 2. No significant difference in erythroid differentiation is apparent between WT and R702C mice.

These analyses are performed three times each in three fetuses. The results are comparable.

Normal progression of erythroid differentiation from BFU-E to RBC was shown by fetal liver cell culture

To estimate erythroid differentiation from the progenitor cells of erythroblasts (Supplemental Fig. 2), BFU-E and CFU-E, Ter119-negative fetal liver cells at E 14.5 were cultured with EPO for two days. Initially, liver cells from E14.5 fetus labeled with anti-Ter119 antibody and anti-CD71 antibody were analyzed by flow cytometric analysis, which did not show significant differences in cell density between WT and R702C (Fig. 2A, 2B).

Fetal liver cells from which Ter119-positive cells had been depleted by microbeads were cultured with EPO for two days and flow cytometric analysis was performed on days 1 and 2. However, no differences in differentiation were observed between WT and R702C (Fig. 2C, 2D). These results indicated that the erythroid differentiation of R702C from BFU-E to RBC was normal in the fetal liver assay.

R702C mice showed comparable ratios of progenitor cells before proerythroblasts in BM, but higher ratios of proerythroblasts and CFU-E in spleen

To estimate erythroid differentiation of progenitor cells of erythroblasts in greater detail using BM and spleen cells (Supplemental Fig. 2), lineage-negative cells were isolated from BM or spleen cells. The c-kit^{mediate-high}Sca-1^{low}CD41^{low}FcγRII/III^{low} cells were then identified by CD150 and endoglin, and divided into the three regions Q1-3, with Q1 containing proerythroblasts and CFU-E, Q2 containing BFU-E, and Q3 containing MEPs.¹⁷ In comparison with WT mice, R702C vav1 mice displayed no significant difference in BM (Fig. 3A, 3B). However, R702C vav1 mice showed a higher ratio of Q1 in the spleen (57.8%) than WT mice (26.0%) (Fig. 3C, 3D).

These results indicated that erythroid differentiation of BM cells was normal for the R702C variant. However, splenic extramedullary erythropoiesis was more enhanced in R702C vav1 mice than in WT mice, which might compensate for the decreased erythroblasts in BM.

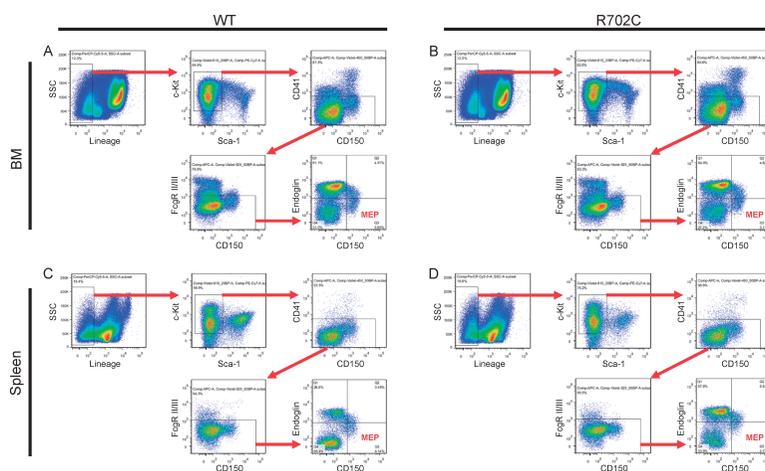


Fig. 3 Flow cytometric analysis of hematopoietic progenitor cells in BM and splenic cells of WT and R702C vav1 mice

The c-kit-positive cells are depleted from BM or spleen cells by magnetic bead selection and analyzed by flow cytometric analysis.

Fig. 3A-3B: Q1 contains proerythroblasts and CFU-E; Q2 contains BFU-E; and Q3 contains MEPs. In BM, no differences are observed between WT and R702C.

Fig. 3C-3D: In the spleen, R702C shows a larger number of cells in Q1 (57.8%) than WT (26.0%). Each analysis is repeated three times and the same results are obtained.

DISCUSSION

MYH9 disorders with R702C are associated with the development of renal failure.¹³ Exclusion of renal anemia should thus be carefully performed. This study generated R702C *vav1* mice expressing the *Myh9* R702C mutation specific to hematopoietic cells. R702C *vav1* mice showed lower levels of Hb and higher levels of EPO compared to WT mice. What was more, in comparison with R702C and R702C *vav1* mice, they displayed almost the same level of Hb, indicating that renal anemia could be excluded as the etiology.

The anemia caused by *Myh9* R702C was characterized by elevated EPO, along with normal levels of reticulocyte ratio and splenomegaly with erythroblasts present at a higher rate than seen in WT mice. These findings seemed to indicate an erythropoietic disorder. A previous report showed that suppression of myosin II by blebbistatin disturbed erythroid differentiation in mice.⁵ Myosin IIA was also reported to play an important role in ring ingression in cytokinesis.⁷ These reports implied that the abnormal myosin IIA affected erythroid cell differentiation by abnormal cytokinesis. To estimate erythroid differentiation, fetal liver and BM cells were subjected to flow cytometric analysis. However, no abnormalities in erythroid differentiation from MEPs to RBCs were obvious.

The involvement of myosin IIA in reticulocyte maturation and RBC membrane structure has been reported by some groups. Ubukawa et al reported that inhibition of non-muscle myosin heavy chain IIB disturbed enucleation, but not myosin IIA,⁸ whereas Moura et al reported that myosin IIA was involved in vesicle clearance during human reticulocyte maturation.¹⁸

To check the enucleation process, blood and BM smears from R702C or R702C *vav1* mice were examined for nucleated red cells, the existence of which could be evidence of disordered enucleation. However, no such cells were observed (data not shown).

What was more, myosin IIA might play an important role in maintaining the biconcave cell shape of RBCs. Smith et al demonstrated that myosin IIA forms bipolar filaments in RBCs and its motor activity regulated interactions with the spectrin-F-actin network to control the biconcave shape and deformability of RBCs.⁹ Thus, RBCs in patients with *MYH9* disorders displayed reduced hemoglobin content and elongated shapes, with significant difference in MCHC and RDW between healthy controls and patients with *MYH9* disorders.¹⁰ However, these findings were not evident in the present study. The osmotic fragility test was also performed to estimate the vulnerability of the RBC membrane, but the results were comparable between R702C *vav1* and WT mice (Supplemental Fig. 3). Of course, these findings were insufficient to completely exclude disorders in the enucleation process or abnormalities of the erythrocyte membrane.

Unfortunately, the mechanism by which R702C caused erythroid abnormality in mice was not revealed in this study. As possible mechanisms remaining to be investigated, myosin IIA is involved in the BM microenvironment. Shin et al reported that myosin IIA was required for sustained engraftment of hematopoietic stem cells and progenitors.⁴ In that study, erythroid differentiation did not show any obvious abnormality. However, the ratio of erythroblasts to total erythroid cells was lower in R702C mice than in WT mice, even though EPO levels were higher in R702C than in WT mice. If R702C causes abnormalities in the BM microenvironment, those results would be reasonable. Whether abnormal myosin IIA affects the BM microenvironment should thus be elucidated as a future task.

In conclusion, *Myh9* R702C causes erythroid abnormality in mice. However, specific abnormalities were unable to be identified in the process of erythroid differentiation from MEPs to RBCs. As one possible mechanism, abnormal myosin IIA may affect the BM microenvironment, and that is considered as a future research topic.

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CONFLICT OF INTEREST

None.

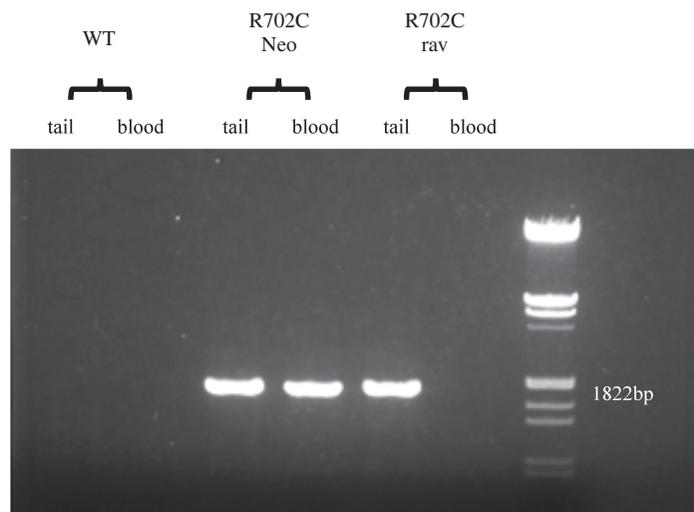
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Myh9 R702C mutation causes anemia in mice**Appendix:** Supplemental data**Supplemental Table 1** The numbers of BFU-E and CFU-E in colony assays

	CFU-E	BFU-E		CFU-E	BFU-E
WT Ex1	264	51.6	R702C vav1 Ex1	180	66
WT Ex2	220	36	R702C vav1 Ex2	200	73.9
WT Ex3	160	51	R702C vav1 Ex3	140	46
WT Ex4	152	51	R702C vav1 Ex4	176	50
WT Ave	199	47	R702C vav1 Ave	174	59
WT SD	53	8	R702C vav1 SD	25	13

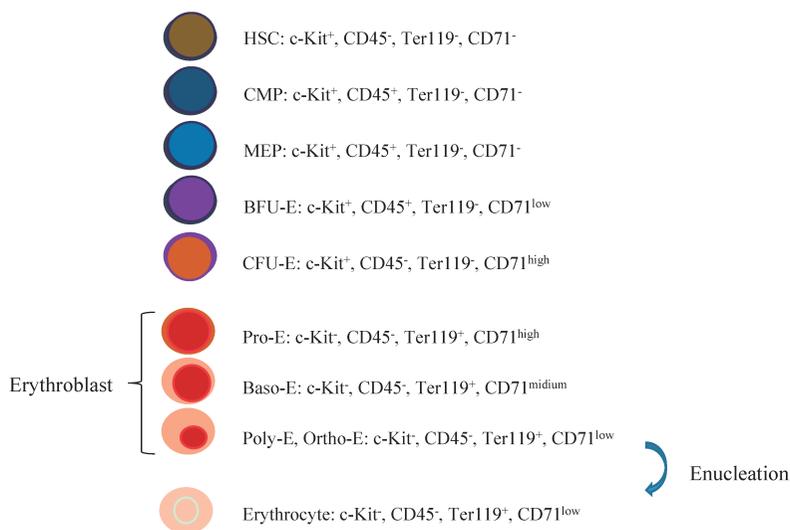
**Supplemental Fig. 1** Generation of R702C vav1 mice

The loxP-neo cassette was removed by crossing heterozygous *Myh9* R702C Neo mice with vav1-Cre mice that express Cre recombinase in the hematopoietic system to yield hematopoietic system-specific *Myh9* R702C heterozygous knock-in mice (R702C vav1 mice).

WT: wild-type mice

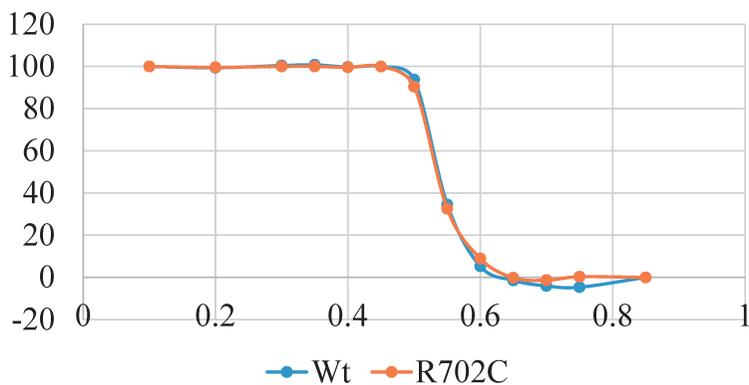
R702C Neo: *Myh9* R702C Neo mice

R702C vav1: *Myh9* R702C vav1 mice



Supplemental Fig. 2 Erythroid differentiation

Region I in Fig 1 contains erythroblasts including proerythroblasts (Pro-E), basophilic erythroblasts (Baso-E), polychromatic erythroblasts (Poly-E) and orthochromatic erythroblasts (Ortho-E). Region II contains erythrocytes. Fetal liver assay (Fig 2) estimated erythroid differentiation from BFU-E to erythrocytes. Flow cytometric analysis of hematopoietic progenitor cells in BM and splenic cells (Fig 3) estimates differentiation from MEP to CFU-E.



Supplemental Fig. 3 Osmotic fragility test

No significant differences exist between WT and R702C *vav1* mice.