## Deletion of miR-451 curbs JAK2(V617F)-induced erythrocytosis in polycythemia vera by oxidative stress-mediated erythroblast apoptosis and hemolysis

Myeloproliferative neoplasms (MPN) are chronic blood cancers characterized by overproduction of blood cells of the erythroid and myeloid lineages. Thrombosis, progression to myelofibrosis, and transformation to acute leukemia represent major causes of morbidity and mortality in MPN patients. Polycythemia vera (PV) is an MPN that presents with erythrocytosis, and is often accompanied by leukocytosis and thrombocytosis.[1] Current treatment for PV focuses on reducing thrombotic risks by phlebotomy and chemotherapy, but not all patients respond (or they are intolerant), and some forms of chemotherapy may increase the risk for leukemic transformation.[2] More than 95% of PV patients harbor an activating driver allele of the tyrosine kinase JAK2, JAK2*V617F*, and thus JAK2 kinase inhibitors have been developed and approved for use in PV. However, these drugs cause dose-limiting cytopenias and toxicity, in part because they do not distinguish between JAK2*V617F* and

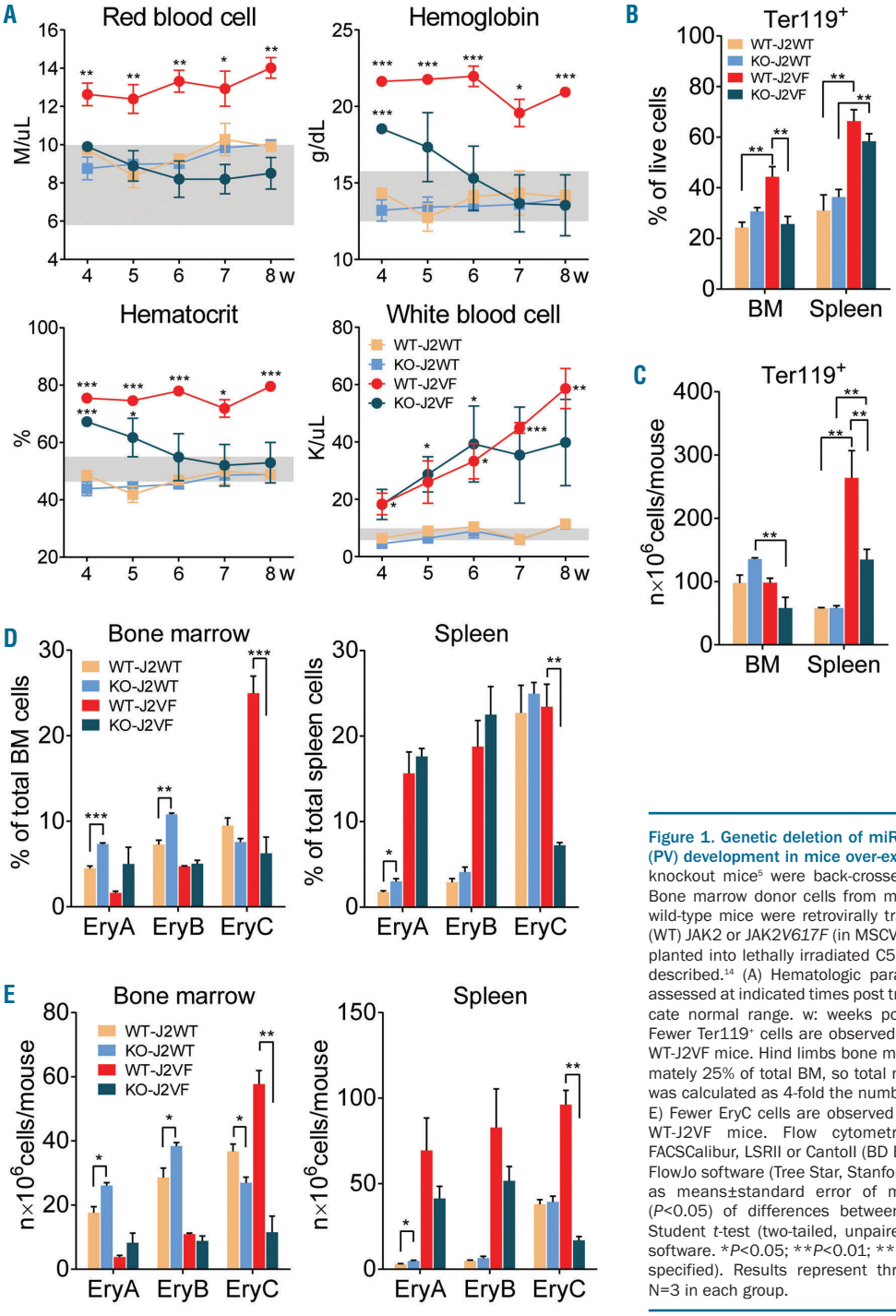


**Figure 1. Genetic deletion of miR-451 curbs polycythemia vera (PV) development in mice over-expressing JAK2*V617F*.** miR-451 knockout mice[5] were back-crossed onto C57BL/6 background. Bone marrow donor cells from miR-451 knockout (KO) mice or wild-type mice were retrovirally transduced to express wild-type (WT) JAK2 or JAK2*V617F* (in MSCV-IRES-GFP vector) before transplanted into lethally irradiated C57BL/6 recipients as previously described.[14] (A) Hematologic parameters of transplanted mice assessed at indicated times post transplantation. Gray zones indicate normal range. w: weeks post transplantation. (B and C) Fewer Ter119$^+$ cells are observed in KO-J2VF mice compared to WT-J2VF mice. Hind limbs bone marrow (BM) represents approximately 25% of total BM, so total number of BM cells per animal was calculated as 4-fold the number from hind limbs BM. (D and E) Fewer EryC cells are observed in KO-J2VF mice compared to WT-J2VF mice. Flow cytometry data were acquired on FACSCalibur, LSRII or CantoII (BD Biosciences) and analyzed with FlowJo software (Tree Star, Stanford, CA, USA). Data are reported as means±standard error of mean. Statistical significance ($P$<0.05) of differences between groups was determined by Student $t$-test (two-tailed, unpaired) using the GraphPad Prism software. *$P$<0.05; **$P$<0.01; ***$P$<0.001 (*vs.* WT-J2WT unless specified). Results represent three independent experiments. N=3 in each group.

wild-type JAK2, and JAK2 is essential for the generation of multiple blood cell lineages and has functions beyond the hematopoietic system.[3] Novel therapeutic strategies are needed that can specifically target PV cells. Here, we show proof-of-principle evidence that inhibition of an erythroid-specific micoRNA, miR-451, specifically restrains erythrocytosis in PV.

MicroRNA (miR) are small non-coding RNA that regulate the translation and stability of target mRNA, thus dictating the production of target proteins. They are often expressed in a tissue and developmental-specific manner, and play important roles in the development and physiology of most tissues. miR-451 is expressed almost exclusively by erythroid cells and promotes terminal erythroid maturation.[4-9] Removal of miR-451 does not significantly affect steady-state erythropoiesis; however, miR-451 knockout sensitizes animals to oxidant-induced hemolysis.[4-6] This sensitivity arises because miR-451 deletion derepresses 14-3-3ζ expression. 14-3-3ζ suppresses FoxO3-induced anti-oxidant gene expression, which protects against reactive oxygen species (ROS). Growing evidence has demonstrated that ROS can stimulate cancer cell proliferation and neoplastic transformation, but high levels of ROS are cytotoxic. JAK2*V617F* induces ROS, and miR-451 is up-regulated both in PV CD34$^{+}$ cells and in the SET2 cell line that expresses JAK2*V617F*.[12,13] We therefore hypothesized that miR-451 inhibition might promote ROS-induced hemolysis to curb JAK2*V617F*-driven erythrocytosis while sparing normal erythrocytes and other blood cells.

To test the hypothesis, we genetically deleted miR-451 in a murine JAK2*V617F*-induced PV model.[14] Bone marrow cells from miR-451$^{-/-}$ or miR-45$^{1+/+}$ littermates were transduced to express either JAK2*V617F* or wild-type (WT) JAK2, then transplanted into lethally irradiated WT recipient mice. As expected, mice reconstituted with miR-451$^{+/+}$ cells expressing JAK2*V617F* (WT-J2VF) developed PV with elevated hematocrits, hemoglobin, red blood cell (RBC) counts, and white blood cell (WBC) counts (Figure 1A). In contrast, mice reconstituted with miR-451$^{-/-}$ cells expressing JAK2*V617F* (KO-J2VF) showed normal RBC parameters after 6-weeks post transplant (Figure 1A). A mild increase in hemoglobin and hematocrit in KO-J2VF mice was observed at earlier time points, which may reflect transient contribution from transduced progenitor cells. Consistent with the fact that miR-451 is almost exclusively expressed in erythroid cells, and thus its deletion was not expected to affect WBC numbers, WBC counts were similarly and significantly elevated in both WT-J2VF and KO-J2VF animals (Figure 1A). As controls, blood parameters of animals receiving either miR-451$^{+/+}$ cells or miR-451$^{-/-}$ cells expressing WT JAK2 (WT-J2WT or KO-J2WT) were similar and in the normal range (Figure 1A). Therefore, deletion of miR-451 specifically curbs erythrocytosis but not leukocytosis in JAK2*V617F*-induced PV development.

We quantified erythroid cells in the bone marrow and spleen using the erythroid lineage marker Ter119. Ter119$^{+}$ erythroid cells were dramatically increased in WT-J2VF animals compared to WT-J2WT mice, particularly in the spleen, consistent with erythrocytosis. In KO-J2VF mice, this increase was significantly reduced (Figure 1B and C). We further categorized erythroid precursors into three subsets with progressive differentiation stages based on the expression of Ter119, the transferrin receptor (CD71), and cell size (FSC): EryA (TER119$^{high}$CD71$^{high}$FSC$^{high}$) for basophilic; EryB (TER119$^{high}$CD71$^{high}$FSC$^{low}$) for late basophilic and polychromatic; and EryC (TER119$^{high}$CD71$^{low}$FSC$^{low}$) for orthochromatic erythroblasts and reticulocytes.[15] In both bone marrow and spleen, KO-J2VF mice showed a dramatic decrease in EryC cells compared to WT-J2VF mice (Figure 1D and E and *Online Supplementary Figure S1*). These results demonstrated that deletion of miR-451 restrained erythroid output in JAK2*V617F*-induced PV, suppressing the expansion of late erythroblast EryC cells. Morphological analysis supported a shift toward earlier erythroblasts in KO-J2VF *versus* WT-J2VF mice (*Online Supplementary Figure S2*).

To examine the mechanisms underlying how deletion of miR451 restrains PV, we measured ROS levels in EryA, EryB and EryC cells of WT-J2VF and KO-J2VF animals. JAK2 or JAK2*V617F* was expressed bicistronically with GFP, so GFP$^{+}$ cells were gated for analyses. ROS levels decreased progressively as erythroid precursors mature due to upregulation of anti-oxidant genes during erythroid differentiation (Figure 2A).[16] Strikingly, the levels of ROS were significantly increased only in KO-J2VF EryC cells but not in earlier erythroblast subsets (Figure 2A and B), consistent with a specific loss of EryC cells in KO-J2VF animals. The increase in EryC ROS levels was not observed in WT-J2VF mice or KO-J2WT mice (*Online Supplementary Figure S3*), indicating a synergistic effect of combining expression of JAK2(V617F) with deletion of miR-451. In line with these results, EryC cells from KO-J2VF mice showed hightened apoptosis compared to those from WT-J2VF mice (Figure 2C and *Online Supplementary Figure S4*).

We next determined whether JAK2*V617F*-expressing RBC are also more sensitive to oxidant-induced hemolysis when devoid of miR-451. We found that GFP fluorescence (the indicator for JAK2*V617F* expression) was not detected in RBC, thus an alternative method was needed to identify WT-J2VF and KO-J2VF erythrocytes. We took advantage of a transgenic mouse line that expresses Kusabira Orange in all tissues including RBC (kindly provided by Dr. Nakauchi).[17] GFP$^{+}$ cells from the bone marrow of WT-J2VF and KO-J2VF mice were sorted and subsequently transplanted into lethally irradiated Kusabira Orange mice. In these secondary recipients, WT-J2VF or KO-J2VF RBC were identified by the lack of Kusabira Orange fluorescence (transplanted donor cells), while residual control RBC from the recipient mice were positive for Kusabira Orange fluorescence (control cells). Consistent with results in EryC cells, KO-J2VF RBC expressed significantly higher levels of ROS in comparison to WT-J2VF RBC, while control RBC showed similarly low ROS levels in both animals (Figure 2D). KO-J2VF RBC also contained fewer cellular free (reduced) thiols (*Online Supplementary Figure S5*). Consequently, upon exposure to hydrogen peroxide ($H_2O_2$), a physiological ROS precursor, KO-J2VF RBC exhibited enhanced hemolysis (Figure 2E).

To gain further molecular insights, we examined known anti-oxidant genes downstream of miR-451.[4] We found that expression levels of *Gpx1* (encoding glutathione peroxidase 1) and *Cat* (encoding catalase) were significantly lower in sorted EryA and EryB cells from KO-J2VF mice compared to those from WT-J2VF mice (Figure 2F). Moreover, sorted KO-J2VF RBC showed reduced catalase protein levels compared to WT-J2VF RBC (Figure 2G). Reduced expression of Gpx1 and Cat may thus contribute to the heightened ROS levels and augmented apoptosis and hemolysis in KO-J2VF cells.

To corroborate the role of ROS in restraining erythrocytosis by miR-451 inhibition *in vivo*, we treated WT-J2VF and KO-J2VF animals with a well established anti-oxi-

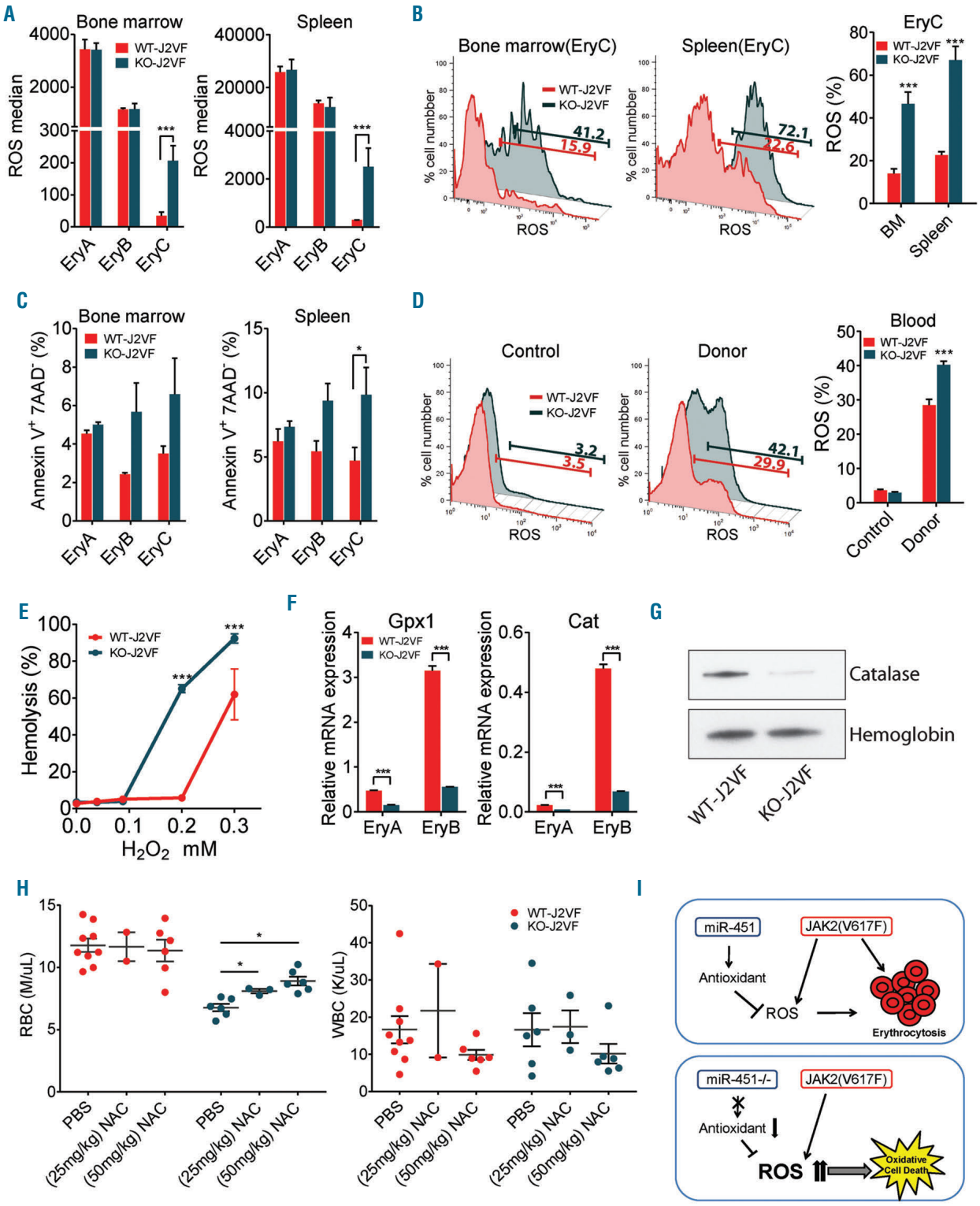


**Figure 2. miR-451 deletion synergizes with *JAK2V617F* in sensitizing reactive oxygen species (ROS)-induced death of late erythroblasts and red blood cells (RBC).** (A and B) Quantitative analyses of ROS in GFP+ erythroblast subsets. Cells were incubated with 2.5 μM CellROX Deep Red reagent (Life Technologies) for 30 minutes (min) at 37°C after surface markers staining. Median fluorescence intensity (MFI) was quantified by flow cytometry in (A). (B) Cell numbers are normalized to mode (highest peak in histogram). (C) Quantitative analyses of Annexin V-binding in GFP+ erythroblast subsets. Apoptosis was quantified by staining with annexin V (BD Pharmingen) and a vital dye (7-AAD, BD Pharmingen). Apoptotic cells were identified as Annexin V+ 7AAD-. (D) Higher levels of ROS are detected in knockout (KO)-J2VF RBC. Cell numbers are normalized to mode. (E) KO-J2VF RBC are more sensitive to $H_2O_2$-induced hemolysis. Erythrocytes were washed, resuspended in PBS with 20 mM glucose, and incubated with various concentrations of H2O2 for 3 hours (h) in a standard tissue culture incubator (37°C, 5% $CO_2$). Hemolysis was quantified by flow cytometry. (F) *Gpx1* and *Cat* gene expression in sorted EryA and EryB cells was quantified by real-time polymerase chain reaction (RT-PCR) analysis. Relative mRNA expression was normalized to actin. (G) Cell lysates from sorted wild-type (WT)-J2VF and knockout (KO)-J2VF RBC were immuno-blotted with antibodies to catalase or hemoglobin β as loading controls. (H) N-acetylcystein (NAC) dose-dependently increases RBC numbers in KO-J2VF mice. 25 or 50 mg/kg NAC were administrated in WT-J2VF or KO-J2VF mice for two days. (I) Model of miR-451 deletion in curbing JAK2*(V617F)*-induced erythrocytosis. *$P$<0.05; ***$P$<0.001.

dant N-acetylcysteine (NAC). NAC treatments in KO-J2VF mice showed a dose-dependent increase in RBC numbers compared to vehicle-treated controls, while no significant difference in RBC numbers was observed in NAC treated WT-J2VF mice (Figure 2H). In parallel, WBC numbers were not affected by NAC treatments (Figure 2H).

Together, these results demonstrated that miR-451 deficiency sensitizes erythroid precursors and RBC to oxidative stress-induced destruction, thereby inhibiting erythrocytosis in PV (Figure 2I). Interestingly, the involvement of ROS in MPN is more complex. In contrast to our short-term NAC treatment, long-term treatment of JAK2*V617F* mice alleviates PV, possibly by eliminating a ROS-mediated pro-survival/proliferative signal in earlier progenitors.[10] These results also do not rule out other mechanisms by which miR-451 might restrain JAK2*V617F*-driven erythrocytosis, such as those that regulate erythroblast maturation. This may explain the increase in apotosis of KO-J2VF EryB cells, which showed similar ROS levels as EryB cells in WT-J2VF animals.

In summary, deletion of the erythroid-specific miR-451 is well-tolerated under normal homeostasis, but in PV erythroblasts and RBC its removal leads to a heightened sensitivity to oxidative stress-induced apoptosis and hemolysis. miRNA inhibitors are currently being explored as therapeutics.[18] We propose that lineage-specific modulators, such as miR-451 inhibitors, may represent a new personalized way in treating PV. It is important to note that PV is a stem cell disease, and miR-451 suppression only impacts late erythroid precursors and RBC. miR-451 inhibitors should thus be used in conjunction with JAK2 inhibitors or other treatments that target the diseased hematopoietic stem cells. That said, the combined therapy may allow lower dosages of the JAK2 inhibitors, thereby lessening cytotoxicity.

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