Erythroid lineage-restricted expression of Jak2V617F is sufficient to induce a myeloproliferative disease in mice

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ABSTRACT

The *JAK2*V617F mutation has been found in most cases of Ph-negative myeloproliferative neoplasms. Recent studies have shown that expression of Jak2V617F in the hematopoietic compartment causes marked expansion of erythroid progenitors and their transformation to cytokine-independence. To determine if erythroid progenitors are the target cells for induction and propagation of Jak2V617F-evoked myeloproliferative neoplasm, we used a conditional Jak2V617F knock-in mouse and an erythroid-lineage specific EpoRCre line. Erythroid-specific expression of heterozygous or homozygous Jak2V617F resulted in a polycythemia-like phenotype characterized by increase in hematocrit and hemoglobin, increased red blood cells, erythropoietin-independent erythroid colonies and splenomegaly. Transplantation of Jak2V617F-expressing erythroid progenitors from the diseased mice into secondary recipients could not propagate the disease. Our results suggest that erythroid lineage-restricted expression of Jak2V617F is sufficient to induce a polycythemia-like disease in a gene-dose dependent manner. Jak2V617F mutation, however, does not confer leukemia stem cell-like properties to erythroid progenitors.

Key words: Jak2V617F, MPNs, erythroid progenitors.

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Introduction

The somatic *JAK2*V617F mutation has been detected in approximately 95% patients with polycythemia vera (PV) and 50-60% patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF).[1-5] Retroviral bone marrow transplantation, transgenic and knock-in mice models of Jak2V617F have shown that Jak2V617F is directly responsible and sufficient to cause PV,[6-11] and may contribute to the pathogenesis of ET and PMF.[7,8,12] Expression of Jak2V617F resulted in marked expansion of erythroid progenitors and Epo-independent erythroid colonies in the bone marrow and spleens of Jak2V617F knock-in mice.[9-11] A previous study indicated that the *JAK2*V617F mutation has an inherent capacity to skew differentiation of PV hematopoietic stem cells (HSC) towards the erythroid lineage.[13] Dupont *et al.* showed that the *JAK2*V617F mutation triggered Epo hypersentivity and erythroid amplification in PV hematopoietic progenitors.[14] Furthermore, enforced expression of *JAK2*V617F in human cord blood hematopoietic progenitors resulted in enhanced erythroid colony formation.[15] Although these studies suggested a direct link between expression of *JAK2*V617F and expansion of erythroid progenitors, it was not clear whether expression of *JAK2*V617F confers leukemia stem cell (LSC)-like properties to erythroid progenitors. In this study, we specifically expressed Jak2V617F in erythroid progenitors using EpoRCre mouse and determined the effects of erythroid-lineage restricted expression of Jak2V617F *in vivo*.

Design and Methods

Mice

Conditional Jak2V617F knock-in,[9] EpoRCre[16] and MxCre[17] mice were as described. The Jak2V617F knock-in mouse was crossed to the EpoRCre mouse to generate EpoRCre;V617F/+ (heterozygous Jak2V617F) and EpoRCre;V617F/V617F (homozygous Jak2V617F) mice. The MxCre;V617F/+ mouse was generated as previously described.[9] Mice with C57BL/6 x 129Sv mixed background were used for all experiments except for transplantation into secondary recipients, in which MxCre;V617F/+ or EpoRCre;V617F/V617F mice were backcrossed to C57BL/6 background for 7 generations. All animal studies were approved by the Committee for the Humane Use of Animals of SUNY Upstate Medical University.

Secondary transplantation

The HSC-enriched Lin-Sca1$^+$c-kit$^+$ (LSK) cells, megakaryocyte-erythroid progenitors (MEP; Lin$^-$Sca1$^-$c-kit$^+$CD34$^-$FcγRII/III$^-$) and granulocyte-monocyte progenitors (GMP; Lin$^-$Sca1$^-$c-kit$^+$CD34$^+$FcγRII/III^{high}) from the BM of the diseased MxCre;V617F/+ mice (12 weeks after induction), and the erythroid progenitors (c-kit^{high}Ter119^{low}CD71^{high} or

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c-kit^{low}Ter119^{high}CD71^{high}) from the BM or spleen of diseased EpoRCre;V617F/V617F mice (12-16 weeks old) were FACS sorted and transplanted along with 10^5 CD45.1$^+$ wild-type BM cells into lethally irradiated (2 x 550 cGy) CD45.1$^+$ recipient mice. Mice were maintained on acidified water.

Colony-forming assay

BM or spleen cells were plated in methylcellulose medium (Stem Cell Technologies) in the presence or absence of Epo. BFU-E colonies were scored on Day 7. CFU-E colonies were counted after two days following staining with benzidine solution (Sigma).

Flow cytometry

BM and spleen cells were stained with either PE- or APC-conjugated monoclonal antibodies specific for Ter119, CD71, CD41, CD61, Mac-1, Gr-1, B220 or Thy-1 (eBioscience, San Diego, CA, USA) for 20 min on ice. Flow cytometry was performed with an LSRII (Beckton-Deckinson, San Diego, CA, USA) and analyzed by using FlowJo software (TreeStar, Ashland, OR, USA).

Statistical analysis

Results are expressed as mean ± SEM, and data were analyzed by the two-tailed Student's t-test. $P<0.05$ was considered statistically significant.

Results and Discussion

The conditional Jak2V617F knock-in mouse[9] was crossed with the EpoRCre mouse which had been shown to cause erythroid lineage-restricted expression of Cre recombinase and efficient recombination of floxed alleles in erythroid progenitors.[16,18] To verify the EpoRCre-mediated expression of Jak2V617F in the hematopoietic compartment, early progenitors and precursor cells of different lineages from the BM of control, EpoRCre;V617F/+ and EpoRCre;V617F/V617F mice were sorted by FACS and PCR was performed. These cells represented HSC-enriched LSK, CMP (common myeloid progenitors), GMP, MEP, erythroid (Ter119$^+$CD71$^+$), myeloid (Gr-1$^+$), megakaryocytic (CD41$^+$CD61$^+$) and B-lymphoid (B220$^+$) cells. EpoRCre induced the deletion of the PGK-Neo-stop cassette and resulted in expression of Jak2V617F specifically in the MEP and Ter119$^+$CD71$^+$ erythroid progenitors in the BM of EpoRCre;V617F/+ and EpoRCre;V617F/V617F mice (*Online*

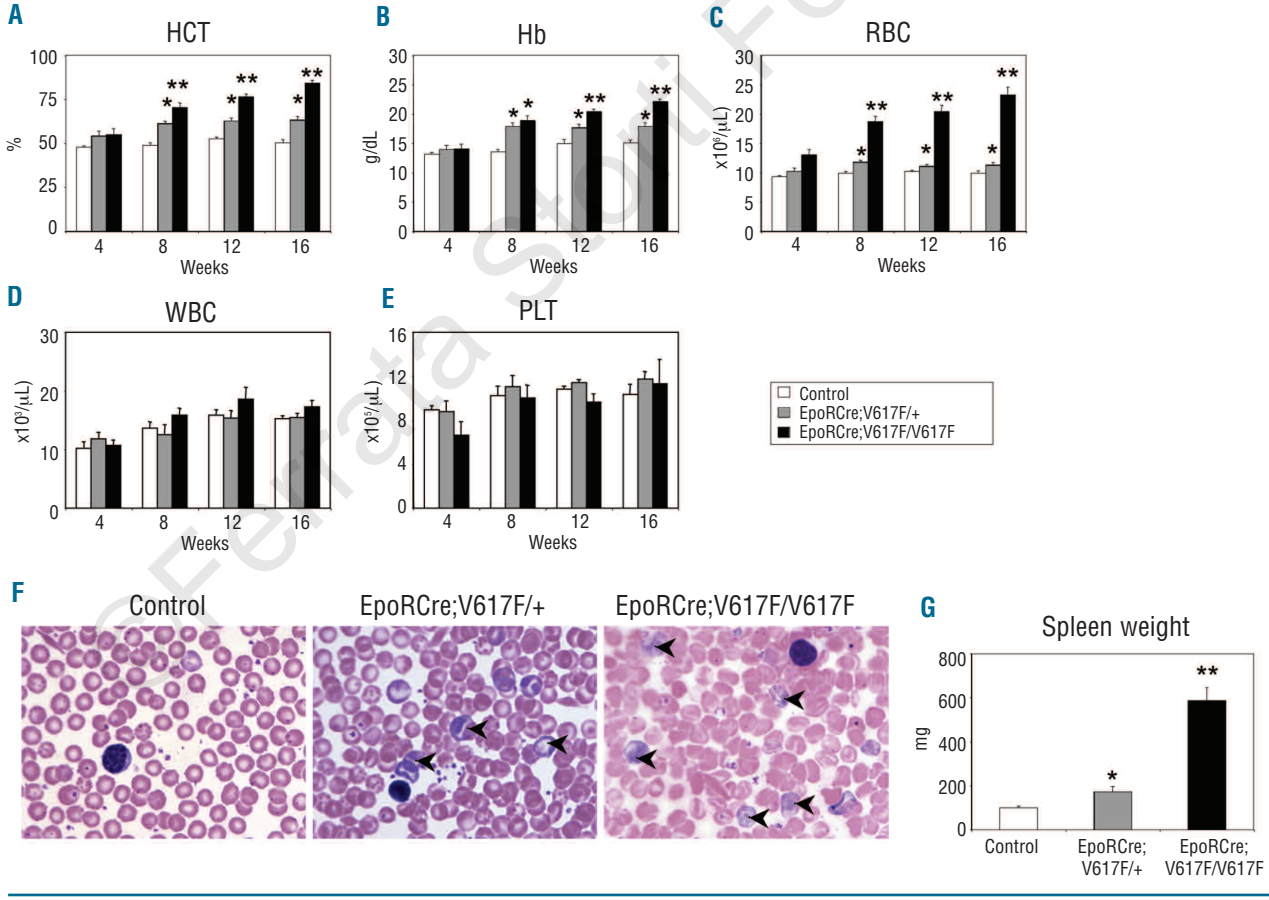

Figure 1. Erythroid lineage-restricted expression of Jak2V617F induces MPN in mice. Peripheral blood (A) hematocrit, (B) hemoglobin, (C) RBC, (D) WBC and platelets (E) were assessed at 4, 8, 12 and 16 weeks after birth in control, Jak2V617F heterozygous (EpoRCre;V617F/+) and homozygous (EpoRCre;V617F/V617F) mice (n=9). (F) Wright-Giemsa staining of the blood smears (1000X) shows increased RBCs and reticulocytes in mice expressing Jak2V617F. Arrowheads point to reticulocytes. (G) Spleen weight was significantly increased in Jak2V617F heterozygous (n=8) and homozygous (n=8) mice compared with controls (n=8). Asterisks indicate significant differences ($P<0.05$) by unpaired, two-tailed Student's t-test (*significance between control and heterozygous, **indicates significance between control and homozygous as well as between heterozygous and homozygous Jak2V617F mice).

Supplementary Figure S1). Notably, EpoRCre did not induce expression of Jak2V617F in the megakaryocytic ($CD41^+CD61^+$) cells (*Online Supplementary Figure S1*) suggesting that EpoRCre expression is mainly restricted to erythroid lineages. To determine the effects of Jak2V617F on erythroid progenitors, three groups of mice were analyzed: V617F/+ (control), EpoRCre;V617F/+ (heterozygous Jak2V617F) and EpoRCre;V617F/V617F (homozygous Jak2V617F). Peripheral blood counts were taken at 4, 8, 12 and 16 weeks after birth. Heterozygous or homozygous expression of Jak2V617F in erythroid progenitors resulted in significant increases in hematocrit, hemoglobin and red blood cells (RBC) in the peripheral blood within eight weeks after birth and increased further over time (Figure 1A-C). Homozygous Jak2V617F expression in erythroid progenitors, however, was associated with a much greater increase in hematocrit, hemoglobin and RBC levels in peripheral blood compared with heterozygous Jak2V617F expression (Figure 1A-C). White blood cell (WBC) and platelet counts in the peripheral blood of these mice were comparable to those observed in control animals (Figure 1D and E). Peripheral blood smears also showed increased RBC and reticulocytes in mice expressing Jak2V617F, with greater increase in homozygous compared to heterozygous mice (Figure 1F). Polycythemia was accompanied by a significant increase in spleen size in mice expressing both heterozygous and homozygous Jak2V617F, although homozygous Jak2V617F expression in erythroid progenitors resulted in a much larger spleen size compared to heterozygous Jak2V617F (Figure 1G). Therefore, Jak2V617F expression in erythroid progenitors induced extramedullary hematopoiesis in mice. Together, these results suggest that erythroid-lineage restricted expression of Jak2V617F induces a myeloproliferative neoplasm (MPN) in mice.

Flow cytometric analysis of the BM and spleen from mice expressing Jak2V617F showed significant increases in $CD71^+Ter119^+$ erythroid progenitors compared with control mice (Figure 2A and B). Expansion of the $CD71^+Ter119^+$ population was greater in the spleen than in the BM of Jak2V617F-expressing mice compared with control animals (Figure 2A and B). Furthermore, homozygous Jak2V617F expression resulted in significantly greater increases in the $CD71^+Ter119^+$ population in the BM and spleens compared with heterozygous Jak2V617F expression (Figure 2A and B). However, erythroid-restricted expression of either heterozygous or homozygous Jak2V617F did not cause a significant increase in myeloid (Gr-1/Mac-1) or megakaryocytic (CD41/CD61) precursors compared with controls (Figure 2A and B). Therefore, the splenomegaly observed upon EpoRCre-mediated expression of Jak2V617F in these animals was mainly due to the expansion of erythroid lineage cells.

Histopathological analyses also showed expansion of erythroid precursors in the spleens of EpoRCre;V617F/+ and EpoRCre;V617F/V617F mice (24-28 weeks old) compared with control animals (*Online Supplementary Figure S2*, left panels). Reticulin staining indicated the absence of fibrosis in the spleens of these animals (*Online Supplementary Figure S2*, right panels). In contrast, MxCre-mediated expression of Jak2V617F in all hematopoietic compartments induced trilineage hyperplasia (expansion of megakaryocytes, erythrocytes and myeloid cells) and fibrosis in the spleens (*Online Supplementary Figure S2*), as we had previously observed.[9]

Erythroid-lineage restricted expression of Jak2V617F also resulted in a marked increase in BFU-E and CFU-E colonies in the BM and spleens in the presence or absence of Epo (Figure 2C and D). Expression of homozygous Jak2V617F, however, resulted in a significantly larger number of BFU-E and CFU-E colonies in the BM and spleens compared with heterozygous Jak2V617F (Figure 2C and D). The presence of a larger number of Epo-independent CFU-E colonies in the BM and spleens of Jak2V617F mice (Figure 2D) suggested that erythroid progenitors were efficiently transformed by Jak2V617F expression. Together, these data establish that erythroid-specific expression of Jak2V617F is sufficient to transform erythroid progenitors and induce MPN in mice.

Although HSCs are frequent targets of transformation in leukemogenesis and have the capacity to initiate the disease,[19,20] several recent studies suggest that more mature progenitor cells, which normally lack any potential for self-renewal, could also be an origin of LSC.[21-23] The oncogenic mutations may confer self-renewal properties to committed progenitors in some myeloid malignancies.[21-23] We have previously shown that the MxCre-mediated expression of Jak2V617F in all hematopoietic compartments resulted in a PV-like disease associated with a marked increase in MEP and erythroid progenitors.[9] We also showed that the MPN disease in MxCre;V617F/+ mice could be transplanted into secondary recipients.[9] To determine whether Jak2V617F expression confers LSC-like properties to committed progenitors (MEP or GMP) or erythroid progenitors, we performed transplantation experiments. We sorted LSK, MEP and GMP from the BM of diseased MxCre;V617F/+ mice, and early erythroid progenitors ($c\text{-}kit^{high}Ter119^{low}CD71^{high}$ or $c\text{-}kit^{low}Ter119^{high}CD71^{high}$) from the BM of diseased homozygous Jak2V617F-expressing EpoRCre;V617F/V617F mice, and transplanted them into lethally irradiated $CD45.1^+$ wild-type recipient animals (*Online Supplementary Figure S3A*). Since EpoRCre-mediated expression of Jak2V617F resulted in marked expansion of the $c\text{-}kit^{low}Ter119^{high}CD71^{high}$ population in the spleens of mice (Figure 2A), we also sorted $c\text{-}kit^{low}Ter119^{high}CD71^{high}$ populations from the spleens of diseased EpoRCre;V617F/V617F mice and transplanted them into recipient animals. We chose to use the EpoRCre;V617F/V617F over EpoRCre;V617F/+ mice for secondary transplantation since the disease was much stronger in homozygous Jak2V617F-expressing EpoRCre;V617F/V617F mice than in the heterozygous EpoRCre;V617F/+ mice. Peripheral blood parameters were assessed at 4, 8, 12 and 16 weeks post-transplantation. Recipient animals receiving LSK developed an MPN phenotype characterized by an increase in hematocrit, hemoglobin and RBC levels in the peripheral blood within four weeks after transplantation and those parameters remained high over 16 weeks (*Online Supplementary Figure S3B*). In contrast, recipients of MEP or GMP from MxCre;V617F/+ mice or erythroid progenitors from EpoRCre;V617F/V617F mice showed no sign of disease and their blood parameters were normal over a period of 16 weeks after transplantation (*Online Supplementary Figure S3B*). Also, the spleen size was normal in the recipient animals receiving GMP, MEP or eyrthroid progenitors (*data not shown*). Analysis of the ratio of Jak2V617F-expressing ($CD45.2^+$) *versus* wild-type ($CD45.1^+$) cells in the peripheral blood and BM of the recipient animals at 16 weeks after transplantation showed that, whereas the majority of the cells in the recipient animals receiving LSK were Jak2V617F-expressing donor-derived ($CD45.2^+$), all the cells in the

peripheral blood and BM of the recipient mice receiving GMP, MEP or erythroid progenitors were wild-type CD45.1$^+$ (*Online Supplementary Figure S3C*), indicating that Jak2V617F-expressing GMP, MEP or eryhtroid progenitors failed to self-renew and could not propagate the disease in the secondary recipients. It should be noted that the erythroid progenitors cannot be tracked by CD45.1/CD45.2 surface markers,[24] but the failure to observe any sign of the disease (as assessed by blood counts; *Online Supplementary Figure S3B*) in secondary recipients strongly indicates that Jak2V617F-expressing erythroid progenitors are not capable of transferring the MPN phenotype. These results suggest

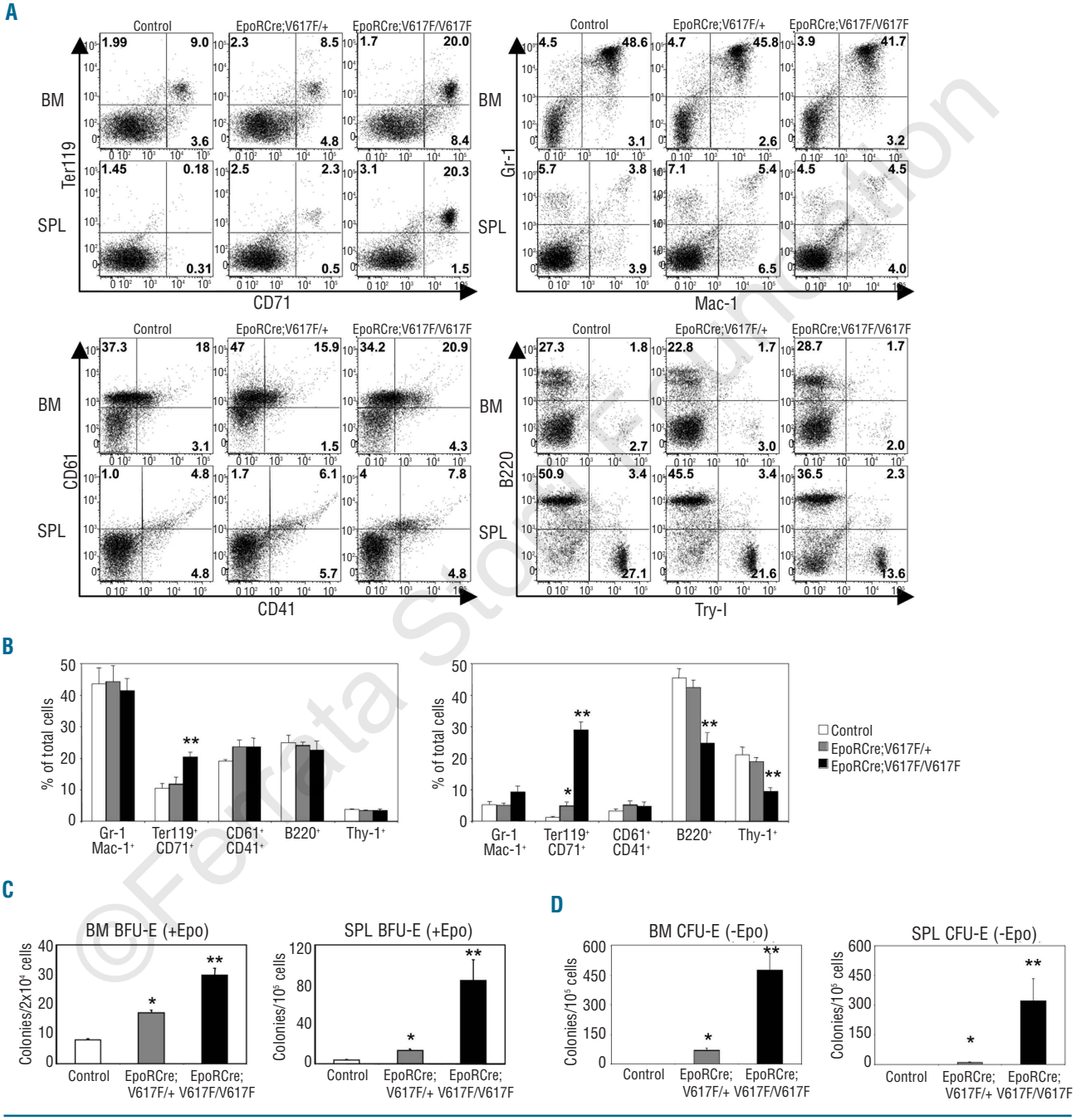

Figure 2. Erythroid-specific expression of Jak2V617F significantly expands erythroid compartment. (**A**) Representative dot plots show a significant increase in CD71$^+$Ter119$^+$ erythroid populations in the BM and spleens of mice expressing heterozygous or homozygous Jak2V617F compared with controls. (**B**) Percentages of myeloid (Gr-1/Mac-1), erythroid (Ter119/CD71), megakaryocytic (CD41/CD61), and lymphoid (B220/Thy-1) populations are shown in histograms. Data from 5 independent experiments are presented as mean ± SEM. (**C** and **D**) BM and spleen cells from control, heterozygous and homozygous Jak2V617F mice were plated in methylcellulose medium in the presence or absence of Epo. BFU-E colonies (**C**) were counted on Day 7. Epo-independent CFU-E colonies (**D**) were counted after two days. Results from 8 independent experiments are presented as mean ± SEM. Asterisks show significant differences by unpaired, two-tailed Student's t-test (*P<0.05 and **P<0.005). Notably, homozygous Jak2V617F expression resulted in significantly greater expansion of erythroid progenitors and Epo-independent CFU-E colonies compared with heterozygous Jak2V617F.

that Jak2V617F expression does not confer self-renewal or LSC-like properties in committed progenitors (MEP, GMP) or erythroid progenitors.

In this report, we demonstrate for the first time that expression of Jak2V617F in erythroid progenitors is sufficient to induce MPN-like disease in mice. Whereas heterozygous Jak2V617F expression in erythroid progenitors resulted in a mild form of MPN, homozygous Jak2V617F expression caused a significant increase in hematocrit, hemoglobin and RBC counts associated with marked expansion of erythroid progenitors in the BM and spleens, and transformation of erythroid progenitors characterized by large numbers of Epo-independent CFU-E colonies (Figures 1 and 2). These results suggest that Jak2V617F homozygosity enhances erythroid expansion and renders erythroid progenitors more Epo-independent. These findings are consistent with the earlier observation that most homozygous JAK2V617F erythroid progenitors in human PV patients were Epo-independent and more sensitive to Epo compared to heterozyzygous JAK2V617F erythroid progenitors.[14] Notably, the phenotypes observed upon erythroid lineage-restricted expression of Jak2V617F in mice were less strong than those observed with MxCre-mediated expression of Jak2V617F in all hematopoietic compartments including HSC.[9] We and other investigators have observed that Jak2V617F-expressing HSCs are capable of initiating and transferring the MPN in secondary and tertiary recipients[11] (Figure 3). The failure to propagate the MPN into secondary recipients that received the GMP, MEP from the diseased MxCre;V617F/+ mice or erythroid progenitors from the diseased EpoRCre;V617F/V617F mice indicate that Jak2V617F does not confer self-renewal capacity to committed progenitors (GMP, MEP) or erythroid progenitors. Our results suggest that both HSCs and erythroid progenitors may be the targets of Jak2V617F but only HSCs have the unique capacity to self-renew and propagate the MPN disease in mice.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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