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Received: June 30, 2023.
Accepted: September 15, 2023.

Citation: Tyler M. Parsons, Aishwarya Krishnan, Wangisa M.B. Dunuwille, Andrew L. Young, Jason Arand, Wentao Han, and Grant A. Challen. Engineering a humanized animal model of polycythemia vera with minimal JAK2V617F mutant allelic burden. Haematologica. 2023 Sept 28. doi: 10.3324/haematol.2023.283858 [Epub ahead of print]

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Engineering a humanized animal model of polycythemia vera with minimal
JAK2V617F mutant allelic burden

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Author contributions: Conceptualization and study design – GAC, TMP, ALY. Experimentation and data acquisition – TMP, AK, WMBD, WH, JA. Data analysis – TMP, GAC. Funding acquisition – GAC. Project administration and supervision – GAC. Manuscript preparation – TMP, GAC.

Running Head: Engineering a human PV model.

Data-Sharing Statement: Data and protocols are available upon request to the corresponding author.

Word Count: 1,500; 3 Main Figures; 3 Supplemental Figures

Article Summary: We used CRISPR/Cas9 to introduce the JAK2V617F mutation into human stem and progenitor cells to engineer a humanized model of myeloproliferative neoplasm. Data show that polycythemia vera pathologies can manifest from a mutant allele burden of less than 5%, and introduction of the JAK2V617F mutation into older age stem cells has predilection for fibrotic progression.

Disclosures of conflicts of interest: G.A.C. has performed consulting and received research funding from Incyte, Ajax Therapeutics and ReNAgade Therapeutics Management not relevant to this work. A.L.Y. has performed consulting for BioGenerator not relevant to this work. T.M.P. has performed consulting for Pillar Patient Advocates and the MPN Research Foundation not relevant to this work. The remaining authors have no financial interest or connection, direct or indirect, to the published work.

Acknowledgments: We thank Dr. Marianna Ruzinova (Washington University School of Medicine) for pathological scoring of reticulin fibrosis and all members of the Challen laboratory for ongoing contributions and critical discussion. We thank the Washington University Musculoskeletal Histology and Morphometry Core for histology, and the Siteman Cancer Center Flow Cytometry core for cell sorting and analysis.

Funding: The Siteman Cancer Center Flow Cytometry core is supported by NIH Cancer Center Support Grant P30CA091842. This work was supported by the National Institutes of Health (HL147978, CA236819 and DK124883) and Research Scholar Grant CSCC-RSG-23-991417-01-CSCC from the American Cancer Society and the Lisa Dean Moseley Foundation (to G.A.C.). T.M.P. is a fellow of the Leukemia and Lymphoma Society. A.L.Y. was supported by NIH T32HL007088. G.A.C. is a scholar of the Leukemia and Lymphoma Society.
Polycythemia vera (PV) is a chronic myeloproliferative neoplasm (MPN) characterized by the over-production of red blood cells. Over 95% of PV patients’ disease is driven by the $JAK2^{V617F}$ mutation. While $JAK2^{V617F}$-mutant mouse models have provided mechanistic insights into PV biology, most of these models present a mutant cell burden much higher than the variant allele frequency (VAF) of $JAK2^{V617F}$ found in PV patients. Thus, current PV mouse models result in a limited understanding of the earliest stages of PV development including what is the minimal mutant cell burden required for disease manifestation. To circumvent these limitations, we developed an engineered model of PV utilizing CRISPR/Cas9 homology directed repair (HDR) to introduce a $JAK2^{V6717F}$ mutation into the endogenous locus of human CD34+ cells. Xenografting targeted cells into NSGS mice recapitulated human PV pathologies in vivo. We used this tool to address two questions – (1) what is the minimum mutant VAF needed to generate PV pathologies, and (2) does the developmental context of the cell of origin influence disease trajectory of MPN. This model provides a valuable pre-clinical tool to test new PV therapies in vivo and an alternative model to study the development and progression of PV when primary patient samples are limited or unavailable.

Myeloproliferative neoplasms (MPNs) are driven by somatic mutations acquired in hematopoietic stem and progenitor cells (HSPCs), characterized by the deviant proliferation of one or more myeloid lineages$^{1,2}$. MPN can present as polycythemia vera (PV; excess erythrocytes), essential thrombocythemia (ET; excess platelets), or myelofibrosis (MF; bone marrow fibrosis). The $JAK2^{V617F}$ mutation is a recurrent driver of MPNs$^{3-5}$. However, the burden of $JAK2^{V617F}$ mutant cells varies widely in patients and can induce clinical phenotypes with very low VAF$^{6,7}$. In PV, over 95% of patients have $JAK2^{V617F}$ as the driving pathogenic mutation, but the mutation burden can be below 3% VAF in some patients$^8$. It is not clear how such a low mutant cell burden can generate MPN pathologies.
Current $JAK2^{V617F}$ mouse modeling strategies utilize retroviral transduction$^{9,10}$, transgenic alleles$^{11}$, or genetic knock-in (KI) models$^{12,13}$. However, most of these models yield high $JAK2^{V617F}$ mutational frequencies that do not accurately reflect the clonal trajectory of PV patients. To overcome the limitations of mouse models, we recently developed methods to transplant CD34+ cells from MPN patients to generate patient-derived xenografts (PDXs). In the case of MF, xenografting patient-derived CD34+ cells is able to propagate the genotypes, phenotypes and key patient pathologies such as reticulin fibrosis in PDXs$^{14}$. However, attempts to generate PDXs from PV patients has been less successful, with poor engraftment and limited numbers of CD34+ cells obtainable from the blood of these patients. To circumvent these issues, here we describe a novel model to study development of human PV employing CRISPR/Cas9 methodology to introduce the $JAK2^{V617F}$ mutation into the endogenous locus of HSPCs obtained from human cord blood (CB) or healthy bone marrow (BM; Supplemental Figure 1A).

For a pilot feasibility study, CB-derived CD34+ cells were nucleofected with CRISPR/Cas9 reagents to introduce the $JAK2^{V617F}$ ("VF") mutation into the endogenous locus. For a negative control, a single-stranded oligo donor nucleotide (ssODN) was designed to introduce a silent mutation at the same amino acid, such that there is no resulting protein change ($JAK2^{V617V}$ = "VV") but a single base genetic variant that serves as a trackable barcode. This engineered system was tested by xenografting a high input of 80,000 nucleofected CD34+ CB cells. Knock-in efficiency was 13-14% for both VF and VV variants (Supplemental Figure 1B). There was high human engraftment in both VF and VV groups in peripheral blood (PB; Supplemental Figure 1C) and BM (Supplemental Figure 1D). $JAK2^{V617F}$ cells exhibited increased engraftment in the spleen (Supplemental Figure 1E), splenomegaly (Supplemental Figure 1F) and propagation of the mutation in the BM (Supplemental Figure 1G).
To model PV driven by low $JAK2^{V617F}$ mutant allele burden, we utilized the KI strategy but with a significantly lower cell input. We also sought to examine how the $JAK2^{V617F}$ mutation can lead to distinct MPN pathologies by testing the hypothesis that developmental context of the cell of origin influences the disease trajectory. CD34+ cells from CB and adult BM (donor ages = 32, 39) were nucleofected as above (Figure 1A). 20,000 nucleofected cells were transplanted intra-tibially into sub-lethally irradiated (2.5 Gy) NSGS mice. $JAK2^{V617F}$ and $JAK2^{V617V}$ donor cells were present at low frequencies in the PB of both recipient groups (Figure 1B,C). The myeloid lineage compartment was increased in hCD45+ PB cells in mice that received the $JAK2^{V617F}$ mutant HSPCs derived from either BM or CB compared to $JAK2^{V617V}$ controls (Figure 1D). Mice transplanted with $JAK2^{V617F}$ mutant HSPCs from both CB and BM donors displayed increased hematocrit, hemoglobin, and platelet counts, suggestive of a PV-like phenotype (Figure 1E). WBC counts were not significantly different (Figure 1E).

Flow cytometric discrimination between human- and mouse-derived erythrocytes showed mice receiving VF cells exhibited a higher percentage of human red blood cells (Supplemental Figure 2A). Interestingly, between weeks 16 and 24 post-transplant, certain recipients of BM-derived $JAK2^{V617F}$ cells displayed a marked decrease in hematocrit, hemoglobin, and platelet counts (Figure 1E), potentially suggestive of disease progression from PV to MF. Spleen weights and human cell engraftment in the spleen were increased in both $JAK2^{V617F}$ cohorts (Figure 1F,G), representative of the splenomegaly often present in MPN patients.

Human cell engraftment in the BM mirrored that of the PB (Figure 2A), although overall cellularity was increased in CB-derived $JAK2^{V617F}$ recipients (Supplemental Figure 2B). Again, the myeloid lineage in the BM was increased in the groups which received $JAK2^{V617F}$-derived cells (Figure 2B). Parallel flow cytometric analysis on mCD45+ BM cells from the same mice showed there were no changes in mouse blood cell lineages between any cohort, suggesting that any observed effect was driven by transplanted human-derived cells (Supplemental Figure 2C). Human HSCs (hCD45+, mCD45-, Lineage-
[CD3/14/16/19/20/56], CD34⁺, CD38⁻, CD45RA⁻, CD90⁺; Supplemental Figure 2D) were detected in both CB- and BM-derived JAK2\(^{V617F}\) cohorts, but not JAK2\(^{V617V}\) recipients (Figure 2C), consistent with our prior studies showing that normal human HSCs do not self-renew in the inflammatory environment of NSGS BM\(^{14}\). JAK2\(^{V617F}\)-mutant BM cells showed increased phosphorylation of STAT3 and STAT5, a canonical feature of MPN patients (Figure 2D). Thus, in addition to reproducibly generating hallmark MPN pathologies (Supplemental Figure 3), this system also produces characteristic molecular features of JAK2\(^{V617F}\)-mutant MPN.

Six months post-transplant, VAF was determined by droplet digital PCR (ddPCR) in whole BM and purified hCD45+ cells from the BM of xenografted mice as previously described\(^{15}\). The VAF of JAK2\(^{V617F}\) in whole BM essentially mirrored overall engraftment (Figure 2E). Within the hCD45+ BM cells, the VAF of VV-targeted cells remained relatively consistent over the transplant period. In contrast, there was a significant increase in the VAF of VF-targeted cells (Figure 2F), demonstrating a competitive advantage for JAK2\(^{V617F}\)-mutant clones.

Reticulin staining of the BM revealed fibrosis in the majority of recipients of BM-derived VF-edited cells (5/7), which was not observed in recipients of CB-derived cells edited with the JAK2\(^{V617F}\) mutation (Figure 3A). No reticulin fibrosis was detected in any recipients of JAK2\(^{V617V}\) control cells (Figure 3B). Histopathology showed the BM from VF-targeted recipients displayed increased megakaryocytes (Figure 3C). Several BM-derived JAK2\(^{V617F}\) recipients had distinctive histopathology, presenting dysmorphic (hyper-lobated, staghorn, and/or cloud-like nuclei) and/or multinucleated (distinct, multinucleated nuclei amidst increased cytoplasm) megakaryocytes (Figure 3D). Dysmorphic megakaryocytes were significantly increased in recipients transplanted with JAK2\(^{V617F}\)-edited cells from either CB or BM, whereas multinucleated megakaryocytes were almost exclusively associated with BM-derived JAK2\(^{V617F}\) cells (Figure 3E). The frequency of multinucleated megakaryocytes strongly
correlated with the degree of reticulin fibrosis in the BM (Figure 3F). Moreover, in BM-derived VF recipients, fibrosis grade correlated with decreasing hematocrit and increased spleen weight (Figure 3F). These pathologies are suggestive of disease progression from PV to MF. This engineered system presents a unique opportunity to study the molecular mechanisms that promote MPN that may lead to these distinct disease trajectories.

In conclusion, we present an engineered humanized \textit{JAK2}^{V617F} KI system wherein a minority of mutant HSPCs initiate MPN in the background of normal hematopoiesis. A burden of mutant cells at low VAF (<5%) was able to induce classical MPN pathologies in NSGS mice, mimicking early-stage human PV. However, it should be noted that due to differences in the biology of NSGS mice, the MPN pathologies are not precise recapitulations of human patients or some genetic mouse models of MPN derived in C57Bl/6 backgrounds. While hematocrit was elevated in NSGS recipients of VF-edited cells, it was markedly lower than that observed in PV patients. Similarly, while splenomegaly was observed, it was not to the same relative degree that can occur in MPN patients. Despite these limitations, this model represents a robust and reproducible tool for the investigation of \textit{JAK2}^{V617F} mutant clone fitness and provides a platform for pre-clinical testing of novel PV interventions. Moreover, the majority of the cohort receiving BM-derived VF-mutated CD34+ cells developed reticulin fibrosis at 6-months. At present, there is no mouse model that reliably models the transformation of PV to MF that we present here in a humanized system.
REFERENCES


FIGURE LEGENDS

Figure 1: Engineering a Humanized Model of PV

A. Knock-in efficiency of VF and VV mutations in CD34+ cells of indicated source determined by next-generation sequencing.
B. Engraftment of human cells in the peripheral blood of NSGS mice determined by flow cytometry.
C. VAF of engineered mutations in the peripheral blood of NSGS mice determined by ddPCR.
D. Lineage distribution of engrafted human CD45+ cells in the peripheral blood of NSGS mice.
E. Blood counts of indicated recipient groups across the experimental time course.
F. Spleen weights of mice receiving indicated human cells. NSGS = age-matched irradiated non-transplanted mice.
G. Engraftment of human cells in the spleens of NSGS mice.

n=5-7 mice per group, data are compiled from two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. Mean ± S.E.M values are shown.

Figure 2: Manifestation of PV Pathologies from a Minimal JAK2V617F Mutant Allele Burden

A. Engraftment of human cells in the BM of NSGS mice.
B. Lineage distribution of engrafted human CD45+ cells in the bone marrow of NSGS mice.
C. Absolute number of human HSCs in BM of NSGS recipient mice for each donor group.
D. Western Blot analysis showing activation of the JAK/STAT pathway in VF targeted cells.
E. Variant allele frequency of engineered mutations in BM of NSGS mice determined by ddPCR.
F. Variant allele frequency of engineered mutations specifically within human cell fractions.

n=5-7 mice per group, data are compiled from two independent experiments. *p < 0.05, ***p < 0.001. Mean ± S.E.M values are shown.

Figure 3: Histopathology of Xenografted Mice

A. Representative BM sections of NSGS mice from indicated groups showing reticulin staining.
B. Quantification of the degree of reticulin fibrosis in BM of recipient mice from indicated groups.
C. Representative histological images of BM sections of NSGS mice from indicated recipient groups.
D. Histopathology showing multinucleated megakaryocytes in BM of a mouse receiving BM-derived JAK2V617F targeted cells.
E. Quantification of the percentage of dysmorphic and multinucleated megakaryocytes (MKs) in the BM of recipient mice from indicated groups.
F. Correlations of BM reticulin fibrosis grade with pathological parameters (non-linear regression) for recipients transplanted with BM-derived JAK2V617F targeted cells. Line of best fit (red) and 95% confidence intervals are shown.

n=5-7 mice per group, data are compiled from two independent experiments. *p < 0.05, ***p < 0.001. Mean ± S.E.M values are shown.
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Supplemental Figure 1: **Engineering a Humanized Model of PV**

**A.** Schematic showing experimental workflow.
**B.** Knock-in efficiency of VF and VV mutations in CB CD34+ cells determined by NGS.
**C.** Engraftment of human cells in the peripheral blood of NSGS mice 24-weeks post-transplant determined by flow cytometry.
**D.** Engraftment of human cells in the bone marrow of NSGS mice 24-weeks post-transplant determined by flow cytometry.
**E.** Engraftment of human cells in the spleens of NSGS mice determined by flow cytometry.
**F.** Spleen weights of NSGS mice receiving indicated human cells.
**G.** Variant allele frequency of JAK2V617F mutation in BM of NSGS mice determined by ddPCR.
Supplemental Figure 2: Manifestation of MPN Pathologies from a Minimal JAK2\textsuperscript{V617F} Mutant Allele Burden

A. Ratio of human erythrocytes (hCD235+) to mouse erythrocytes (mTer119+) in the peripheral blood of NSGS mice 24-weeks post-transplant.
B. Total bone marrow counts of NSGS mice from indicated groups 24-weeks post-transplant.
C. Lineage distribution of mouse hematopoietic cells (mCD45+) in BM of NSGS mice 24-weeks post-transplant.
D. Representative flow cytometry gating scheme to identify human HSCs in the BM of NSGS recipient mice.

\(n=3-7\) mice per group, data are compiled from two independent experiments. *\(p<0.05\), ***\(p<0.001\). Mean ± S.E.M values are shown.
Supplemental Figure 3: Experimental Reproducibility

A. Reproducibility of biological features between different experimental cohorts and donor material. Shown are the average values for stated parameters for mice from three distinct experimental cohorts (diamond = pilot study, circle = cohort 1, square = cohort 2).

B. Consistency of hematocrit (HCT) levels in NSGS mice between different experimental cohorts and donor material. Shown are the longitudinal HCT values for each mouse transplanted with the indicated donor cells (grey lines) surrounding the average for all mice within that group (thick colored line).

n=1-4 mice per group, data are compiled from three independent experiments. Mean ± S.E.M values are shown.