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Cytopenia induction by 5-fluorouracil identifies thrombopoietic mutants in sensitized ENU mutagenesis screens

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Abstract

The ability of random mutagenesis techniques to annotate the mammalian genome can be hampered due to genetic redundancy and compensatory pathways that mask heterozygous mutations under homeostatic conditions. The objective of this study was to devise a pharmacologically sensitized screen using the chemotherapeutic drug, 5-fluorouracil (5FU), to induce cytopenia. 5FU dose was optimized in the *129/SvImJ*, *C57BL/6J*, *BALB/cJ*, and *C3H/HeJ* strains of laboratory mice. *N-ethyl-N-nitrosourea* (ENU) mutagenesis was performed on *129/*

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Conflict of interest disclosures

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

SvImJ males and phenotypic variants were identified by backcrossing on to the *C57BL/6J* background. G1 animals were challenged with 100 µg/g 5FU and phenodeviants with altered platelet recovery were monitored. Of 546 G1 animals tested, 15 phenodeviants were identified that displayed increased baseline platelet number, a platelet overshoot, or delayed platelet recovery, thereby demonstrating the utility of this approach for uncovering mutations in megakaryocyte and platelet development. Four G1 mice were selected for further analysis. The phenotypes were heritable in all four strains and genetic mapping identified a chromosome location in two of the three G2 lines tested. In conclusion, our group has developed a sensitized random mutagenesis screen utilizing 5FU and has shown that the strain combination of 129/SvImJ × C57BL/6J is robust for identification of founder lines with defects in megakaryocyte and platelet development.

Most developmental processes are regulated by transcriptional and signaling pathways that are connected through regulatory networks [1–3], which can have functional redundancy. Generally, recessive mutations fail to exhibit a heterozygous phenotype because many genes are haplo-sufficient. However, in a mutant or sensitized background, the threshold for identifying new mutations affecting the regulatory network is lower, allowing for identification of mutations in genes whose function may be masked by redundancy or essential for other developmental processes. Sensitized screens have been used extensively in *Drosophila* to identify roles for essential genes in developmental signaling pathways [4]. Similarly, *p53*-null mice and *W(c-kit)* mutant mice are often crossed with strains null in other genes to identify novel functions of those genes [5,6].

In this report, we describe a sensitized *N-ethyl-N-nitrosourea* (ENU) screen that has generated mutations that affect thrombopoiesis. 5-fluorouracil (5FU) inhibits thymidine synthase, affecting DNA synthesis and possibly RNA synthesis, killing cells in various stages of the cell cycle [7]. 5FU was selected as the pharmacological sensitizing agent because it specifically targets dividing cells, such as the early progenitors of the hematopoietic system, inducing hematopoietic stress. 5FU has dramatic effects on several hematopoietic lineages, inducing a transient myeloid cytopenia, which result in mobilization of quiescent hematopoietic stem cells. Although recovery from 5FU has been documented in *C57BL/6J* [8] and *C3H/HeJ* [9] mice, these studies used different assays to analyze the response. Therefore, there is a need to perform standard assays of multiple strains challenged by 5FU.

Recovery from 5FU treatment has been used to successfully identify novel hematopoietic progenitor phenotypes from heterozygous and homozygous knockout mice [10–14]. In contrast, our goal was to use 5FU-induced cytopenia in a forward genetic screen to increase the efficiency of our screen for novel hematopoietic mutations. Here, we focus on mutations that affect thrombopoietic recovery from 5FU treatment. Using our standardized assays, we performed a sensitized screen utilizing a 5FU-treated cohort of G1 offspring from ENU mutagenized 129/SvImJ male mice bred to *C57BL/6J* females. This strain combination is optimal for elucidation of defects within the megakaryocyte and platelet lineage. We identified four heritable mutants that exhibit defects in thrombopoietic recovery from 5FU treatment.

Materials and methods

Mouse strains

Congenic strains of 129/SvImJ, BALB/cJ, C3H/HeJ, and C57BL/6J were used at 5 to 7 weeks of age to assess hematopoietic recovery. All mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained in a pathogen-free mouse facility at the Toronto Centre for Phenogenomics (Toronto, ON, Canada). All mouse experiments received

approval from the local Animal Care Committee and were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Cytopenia induction

5FU (Sigma, St Louis, MO, USA) was dissolved in phosphate-buffered saline (Gibco, Carlsbad, CA, USA) at a concentration of 15 µg/mL, and then injected intraperitoneally (IP) at the doses indicated (100, 120 and 150 µg/g).

Hematologic analysis

Peripheral blood (20–30 µL) from the saphenous vein was collected with EDTA-coated capillary pipettes (Drummond, Bromall, PA, USA). Complete blood counts were performed with a Coulter Ac-T Differential Hematology Analyzer with veterinary software (Beckman Coulter, Brea, CA, USA). All mice were bled on day 0 (D0) to obtain a baseline, and then once every 4 days unless otherwise stated.

Early progenitor (c-Kit⁺, Thy1^{lo}, Lin⁻, and Sca-1⁺ [KTLS]) analysis

Single-cell suspensions were prepared from the bone marrow (BM) of *C57Bl/6J* female mice. Red blood cell lysis, cell counts, and viability analysis were performed. Twelve million cells were stained with unconjugated rat antibodies specific for lineage (Lin) markers. Cells were washed and lineage-positive cells separated by immunomagnetic labeling (Dynabeads; Invitrogen, Carlsbad, CA, USA). The remaining cells were stained by a phycoerythrin-Texas Red goat anti-rat IgG polyclonal antibody (Invitrogen) and with allophycocyanin-conjugated anti-c-Kit, phycoerythrin-conjugated anti-Sca-1, and fluorescein isothiocyanate-conjugated anti-Thy1.2. Cells were then analyzed by flow cytometry using a modified BD LSR II (Becton Dickinson, Mountain View, CA, USA).

In vitro clonogenic assays

Clonogenic assays were performed as described previously [5,15]. Briefly, red blood cell lysis, cell counting, and viability analysis was performed on BM or splenic single-cell suspensions. Three aliquots of 2×10^4 BM cells (or 1×10^5 spleen cells) were mixed with the appropriate methylcellulose media, plated, and incubated at 5% CO₂, 37°C for each assay. All colony-forming unit in culture (CFU-C) and CFU-erythroid (CFU-E) cultures were grown in methylcellulose M3434 and M3334, respectively (STEMCELL Technologies, Vancouver, BC, Canada). CFU-E precursors were assayed after 2 days by staining in situ with benzidine (Sigma) to detect hemoglobin. After 7 to 10 days of incubation, burst-forming unit-erythroid (BFU-E) and CFU-C were counted based on colony morphology.

Ploidy analysis

BM cells were stained with fluorescein isothiocyanate-conjugated anti-CD41 or rat IgG₁ isotype control for 30 minutes, then washed and incubated in 0.05 µg/mL propidium iodide in 0.1% trisodium citrate at 4°C overnight. Cells were then washed and incubated with 50 µg/mL RNase A for 30 minutes before analysis. Samples were analyzed by flow cytometry on a Beckman Coulter FC500. Megakaryocytes were identified as CD41-positive cells, and propidium iodide staining intensity was used as a measure of DNA ploidy. Each time point had a set of control mice (*C57Bl/6J*, n=3) that were sham-injected (200 µL phosphate-buffered saline IP).

ENU mutagenesis and G1 screen

ENU treatment of *129S1/SvImJ* male mice consisted of either one IP injection of ENU (150 mg/kg) or two IP injections of ENU (100 mg/kg) given 1 week apart. ENU-injected males

were designated G0 males. An F1 generation (*B6;129S*) was produced by breeding G0 males to *C57BL/6J* females, known as generation 1 (G1). Five-week-old G1 animals underwent 5FU treatment to identify defects in platelet (PLT) recovery. Specifically, G1 mice were bled on D0, injected with 5FU (100 µg/g), and then blood was collected on day 6, 8, and 13. Phenodeviant (outliers) G1 mice were identified by comparing their 5FU recovery kinetics to that of 5FU-treated control mice (F1 *129S1/SvImJ* × *C57BL/6J* hybrid no ENU treatment [B6129SF1]). G1 outliers were bred to *C57BL/6J* mice, which produced G2 mice that underwent heritability testing and genome scanning. A phenotype of an ENU line was considered heritable or heritable-low penetrance if 35% to 50% or 10% to 35%, respectively, of G2 mice showed the original phenotype of the G1 parent. To identify the mutation and maintain the line, affected mice were bred to *C57BL/6J* mice producing subsequent generations (G n). Reference values were derived as follows: G2 mice compared to F2 controls (B6;CgN2: B6129SF1 × *C57BL/6J*), G3 mice compared to F3 controls (B6;CgN3: B6;CgN2 × *C57BL/6J*), and G4-10 to *C57BL/6J* controls.

Genetic mapping

DNA from G2 and G3 outliers was isolated and a genome scan was performed using the Mouse MD linkage panel on the GoldenGate SNP (Illumina, San Diego, CA, USA) genotyping platform (Toronto Center for Applied Genomics). Additional backcrosses were performed between phenodeviants and *C57BL/6J* controls to further refine the interval. A high-density single-nucleotide polymorphism map of the specific chromosomal region was created with data from the following databases: Jackson Labs Mouse Genome Informatics, National Center for Biotechnology Information, and Ensembl.

Statistical analysis

Dose, strain, and sex recovery data were combined for two or more groups of animals to represent a single response. Samples containing clots or sampling errors were removed from analysis.

Recovery curves following 5FU treatment were analyzed using one-way repeated measures analysis of variance (ANOVA). A Dunnett's post-test was performed to determine at which time point the parameters significantly changed from baseline. Each group (dose, strain, or sex) was analyzed by two-way repeated measures ANOVA, which compared all two-group combinations to determine whether significant differences exist between a pair of groups (e.g., *Balb/C* vs *C57BL/6*). The pair-wise comparison was followed with a Bonferroni post-test to determine when during the 2-week time course differences existed. To compare nadir or peaks that occur at different time points between two groups, a *t*-test with two-tailed distribution assuming unequal variance (Welch's correction) was performed, while three or more groups were analyzed with a one-way ANOVA.

Data from KTLS populations were analyzed by a *t*-test with two-tailed distribution assuming unequal variance (Welch's correction). Bone marrow and spleen colony assays were analyzed with a one-way ANOVA, followed by a Bonferroni's post-test to detect differences between two time points.

All statistical analysis was performed using GraphPad Prism software. A *p* value 0.05 was accepted as significant in all statistical tests, including *t*-tests, ANOVAs, and post-tests.

Results

Effect of 5FU dose on recovery kinetics

A dominant screen requires that G1 animals remain viable and yet the phenotype must be robust enough to reduce false positives. Thus, we examined the recovery kinetics of red blood cells (RBC), PLT, and white blood cells (WBC) to identify optimal 5FU dose. We examined the recovery kinetics of RBC, PLT, and WBC in *C57BL/6J* female mice at three doses of 5FU: 100, 120, and 150 $\mu\text{g/g}$ (Fig. 1A). All doses of 5FU tested induced anemia of varying severity, 100 $\mu\text{g/g}$ induced a relatively mild anemia, similar levels of anemia were induced at 120 $\mu\text{g/g}$ and 150 $\mu\text{g/g}$ 5FU. Only 150 $\mu\text{g/g}$ 5FU was lethal in 5% of *C57BL/6J* mice.

All doses of 5FU resulted in thrombocytopenia followed by a multifold overshoot in PLT numbers compared to baseline levels (Fig. 1A). The PLT recovery curves were similar for 5FU doses 100 $\mu\text{g/g}$ and 120 $\mu\text{g/g}$, with comparable declines in PLT number and PLT overshoot. However, the platelet recovery pattern following 150 $\mu\text{g/g}$ 5FU was unique, as it induced more extreme and prolonged PLT overshoot.

No 5FU dose tested caused a significant decrease in WBC number (Fig. 1A), but all doses led to WBC overshoot. The degree of WBC overshoot was similar between 100 and 120 $\mu\text{g/g}$ 5FU; 150 $\mu\text{g/g}$ 5FU induced a more extreme WBC overshoot. The WBC overshoot was attributed to elevated proerythroblasts and not neutrophils or lymphocytes (data not shown), because proerythroblasts can be mistakenly identified as WBC based on size similarity by the hematology analyzer.

A 5FU dose of 120 $\mu\text{g/g}$ was chosen as an optimal dose because it caused no lethality in wild-type mice but was still effective at inducing anemia, thrombocytopenia, and PLT overshoot.

Strain-specific effects of 5FU administration

Successful mapping of an ENU mutation requires that both the mutagenesis strain and the mapping strain have similar readouts in phenotyping assays. It is known that the hematological properties of mouse strains are distinct, yet a direct comparison of the effects of 5FU on different mouse strains is not available. Therefore, we wished to investigate potential differences in 5FU recovery kinetics among four commonly used laboratory strains *C57BL/6J*, *BALB/cJ*, *C3H/HeJ*, and *129S1/SvImJ*.

We confirmed that the baseline values for RBC, PLT, and WBC were significantly different between the four mouse strains. 5FU was effective at inducing anemia in all mouse strains tested, but the severity of anemia, timing of the RBC nadir, and subsequent recovery were distinct (Fig. 1B). Specifically, the timing of RBC nadir was different among the strains: D4 for *C3H/HeJ*, D10 for *129S1/SvImJ* and D12 for *C57BL/6J* and *BALB/cJ*. RBC recovery kinetics were highly similar between *C57BL/6J* and *BALB/cJ* mice. Although *129S1/SvImJ* mice had similar RBC recovery kinetics compared to *C57BL/6J* and *BALB/cJ* mice, they became less anemic and recovered more quickly. Strikingly, RBC recovery kinetics were most altered in *C3H/HeJ* mice, as 5FU induced a milder anemia with a rapid recovery.

Although 5FU-induced thrombocytopenia and PLT overshoot occurred in all strains, significant differences were observed in timing and degree of PLT overshoot. For example, the PLT nadir of *C57BL/6J* mice occurred later (D8) than the other strains (D4). Overall, the induction of thrombocytopenia was comparable between *BALB/cJ* and *129S1/ImJ* mouse strains and the timing and degree of PLT overshoot was equivalent between *C57BL/6J*, *BALB/cJ*, and *129S1/ImJ*. A distinctive pattern of PLT recovery was observed in *C3H/HeJ*

mice, as this strain recovered more quickly from thrombocytopenia and had a reduced PLT overshoot.

Three of the mouse strains (*C57BL/6J*, *BALB/cJ*, *129S1/ImJ*) lost 40% to 60% of their WBC mass after 5FU treatment, between D8 and D10, but only *C57BL/6J* and *BALB/cJ* overshoot baseline values. Although WBC recovery patterns of *C57BL/6J*, *BALB/cJ*, and *129S1/ImJ* mice were similar, statistically significant differences exist between the magnitude and duration of WBC overshoot. *C3H/HeJ* mice had a unique WBC recovery pattern compared to the other strains, as WBC nadir (D4) and overshoot (D8) occurred much earlier.

Effects of hematological stress agents on progenitors in the BM and spleen

5FU affects multiple lineages, including progenitor cells, therefore KTLS cells were monitored following 5FU administration in *C57BL/6J* mice. KTLS are a heterogeneous group of hematopoietic cells that contain stem cells and early progenitors. Although the total frequency of KTLS cells was unchanged 18 hours after 5FU treatment (Fig. 2), a subpopulation of c-Kit^{hi} cells was greatly reduced (76–88%). A three- to fivefold increase in KTLS cell frequency was observed 1 week after 5FU treatment, which returned to baseline levels 2 weeks after treatment.

5FU alters BM cellularity following treatment in *C57BL/6J* mice (Fig. 3A). BM cellularity dropped 18 hours after 5FU treatment and continued to decrease to 40% of baseline values until day 7. Within 2 weeks of 5FU treatment BM cellularity had returned to near-baseline values.

Within 18 hours of 5FU treatment, CFU-C frequency dropped by >60% and this change affected all progenitor types (Fig. 3B). After 1 week, CFU-C frequency in the BM increased above baseline values more than twofold, and affected all colony types except BFU-E. The increased CFU-C numbers continued until day 14 and were attributed to a significant overshoot of CFU-granulocyte and CFU-granulocyte macrophage. All other colony types had returned to baseline within 2 weeks.

5FU significantly influenced BM CFU-E frequency, causing a 97% drop in CFU-E 18 hours after 5FU treatment (Fig. 3C). Baseline values were restored within 7 days. No CFU-E overshoot was observed in the given time period.

5FU causes changes in extramedullary hematopoiesis in the spleen of *C57BL/6J* mice, assessed by CFU-C frequency in the spleen over a 2-week period (Fig. 3D). Splenic CFU-C values dropped 90% within 18 hours of 5FU treatment, then increased to near baseline levels after 1 week, and finally overshoot to 4.2-fold baseline levels after 2 weeks. The changes in extramedullary hematopoiesis caused by 5FU were due to changes in both red (BFU-E) and white (CFU-granulocyte; CFU-macrophage; CFU-granulocyte, erythrocyte, monocyte, megakaryocyte; and CFU-granulocyte macrophage) colonies. 5FU led to a 95% loss in red colonies 18 hours post-injection, which did not return to near normal levels until 2 weeks post-injection. White colonies were also reduced by 90% 18 hours after treatment. Two weeks after 5FU treatment white colonies overshoot baseline values by 6.2-fold.

Effect of hematological stress agents on megakaryocyte ploidy

Megakaryocyte ploidy was examined in the BM of 5FU-treated *C57BL/6J* mice by examining the propidium iodide intensity of CD41⁺ cells as a measure of DNA ploidy. We stratified immature megakaryocytes with a DNA content of 2n, 4n, and 8n into Region 1 (R1) and mature megakaryocytes with a DNA content 16n and higher into region 2 (R2). 5FU induced changes in the frequency of CD41⁺ cells and ploidy profiles over the 2-week

time course. 5FU caused a shift in megakaryocyte ploidy within 4 days of treatment, marked by an increase of cells in R1 and a decrease of cells in R2 compared to controls (sham-injected *C57BL/6J*; Fig. 4A and Table 1). The frequency of CD41⁺ cells increased 7 days after 5FU treatment, but returned to normal within 2 weeks of cytopenia induction (Fig. 4B).

5FU G1 screen

A 5FU-sensitized screen was implemented into an ongoing dominant ENU screen, in which the dose of ENU had been optimized in *129S1/Sv1mJ* male mice. Although *BALB/cJ* and *C57BL/6J* mice had the highest degree of concordance in the 5FU recovery curves of RBC, PLT, and WBC, we chose the *129S1/Sv1mJ* and *C57BL/6J* strain combination because we had optimized the dose of ENU in *129S1/Sv1mJ* males and the recovery curves for *129S1/Sv1mJ* and *C57BL/6J* mice were similar. The design of the 5FU-sensitized screen included mutagenesis of *129S1/Sv1mJ* male mice with ENU, which were bred to *C57BL/6J* females, to produce G1 progeny for screening (Fig. 5A).

Five-week-old G1 mice were challenged with 100 µg/g 5FU and RBC, PLT, and WBC recovery was monitored. Comprehensive recovery curves were generated with F1 control mice (*129S1/Sv1mJ* × *C57BL/6J*) (blue line, Fig. 5B). The 5FU recovery curves were then used to select testing days that were optimal for detection of RBC, WBC, and PLT delayed recovery as well as PLT overshoot; D0, D6, D8, and D13 (black line, Fig. 5B).

We examined the effectiveness of 100 µg/g 5FU at inducing cytopenia uniformly in the dominant screen by performing a post-screen analysis to monitor the recovery of RBC, PLT, and WBC in G1 male mice (Fig. 6A). Positive values, or values above the red line, represent a time period when counts for a given parameter were increasing. Negative values, or values below the red line, represent a period of time when counts for a given parameter were decreasing. The RBC recovery in G1 males was variable, particularly between D6 and D8 when 61% of G1 males showed a decrease in RBC and became more anemic, while 39% of G1 males initiated recovery from anemia. Overall, the PLT recovery from 5FU-induced thrombocytopenia occurred in unison, particularly between D0 and D6, as well as D6 and D8. Discord in PLT recovery was observed from D8 to D13 as the overshoot in some mice was increasing, but PLT values returned to normal in other mice. Before 5FU injection, WBC values were highly variable. For example, WBC values in G1 males were 12.6×10^9 /L with a large standard deviation (SD) of 3.0×10^9 /L. After 5FU administration, WBC recovery was relatively uniform throughout the 5FU test.

A total of 546 G1 mice were tested in the 5FU screen (Table 2). We focused our attention of PLT phenotypes because of concerns regarding differences in RBC and WBC recovery parameters leading to false-positive phenotyping. Fourteen phenodeviants were identified and classified as either delayed PLT recovery or PLT overshoot phenotypes. Eleven G1 phenodeviants demonstrated increased PLT overshoot at D13 (*z*-score = 2.75). This included four female (Fig. 6B) and seven male G1 animals (Fig. 6C). Delayed PLT recovery mice were selected using the following criteria: a PLT decrease at each time point between D0 to D8 and a drop from D6 to D8 >2 SD below the average. One female G1 founder (Fig. 6D) and two G1 male mice (Fig. 6E) were identified with this phenotype. Additionally, we found one G1 outlier with high PLT on D0, but a normal 5FU recovery.

Analysis of G2 mice

Four phenodeviant G1 mice were selected for further assessment (Table 2). To determine heritability of a pheno-deviant trait, G1 outliers were bred to a *C57BL/6J* of the opposite sex and their offspring were assessed to determine heritability. A phenotype was designated as heritable if the offspring from the mating displayed the same phenotype as the G1 founder.

In a dominant screen, a phenotype is fully penetrant (100% penetrance) if 50% of the offspring have the same phenotype as the G1 founder. Genome scans using a panel of 750 informative single-nucleotide polymorphisms across all 19 autosomes and the chromosome X were performed on heritable lines to map the mutation to a specific chromosomal location. The 7321 G1 founder was bred to test heritability-based increased PLT overshoot (z -score = 3.96), but also showed delayed RBC recovery (z -score = -2.74) and increased WBC overshoot (z -score = 5.74). The PLT overshoot phenotype was heritable with low penetrance (Fig. 7A). The RBC phenotype was 35% penetrant and the WBC phenotype was 25% penetrant. Hence, the sperm were archived for future analysis of 7321 RBC mutants. Strain 7324 presented with delayed PLT recovery (Fig. 7B), which was heritable with low penetrance (20%). The DNA from eight G2, G3, and G4 mice were used in a genome scan and all eight mice were mapped to five distinct chromosome locations (Ch. 1, 5, 7, 13, and 19). The 7324 strain was subsequently archived for future analysis of the complex mapping. In contrast, the other two strains (7323 and 7325) were heritable and had clear single map locations.

The founder of strain 7323 presented with elevated platelets on D0 (PLT $1699 \times 10^9/L$; z -score = +3.13 SD). The high PLT phenotype of 7323 was shown to be heritable at 30% penetrance with an average PLT value of $1585 \times 10^9/L$; SD = $76 \times 10^9/L$ (F2 Controls $1183 \times 10^9/L$; SD = $20 \times 10^9/L$, Fig. 7C). Eight G2 and G3 mice were used in a genome scan, and all mice mapped to a 24-Mb region at the telomeric end of chromosome 11. Subsequent backcrosses have narrowed the interval to a 10.3-Mb region.

Strain 7325 presented with PLT overshoot and was found to be heritable with 19% penetrance. A genome scan was performed on the DNA from nine G2 and G3 outliers and all mice mapped to a 45-Mb region at the distal end of chromosome 11. The interval encoding the 7325 mutation has been narrowed to an 8.84-Mb region on chromosome 11. The PLT recovery of all G2 females from the 7325 mouse line is shown in Figure 7D.

Discussion

We have utilized ENU in a forward genetic screen to generate new mouse models of human hematopoietic disease, and with mutations affecting developmental pathways in hematopoiesis [15,16]. Although a high percentage of human diseases are dominant or semi-dominant, most mutations eliciting phenotypes affecting the development of hematopoietic lineages are recessive. Consistent with this observation, we found a lack of heritable mutations affecting multilineage and erythroid development in our dominant screen [15]. Thus, we posited that a sensitized screen would enable us to identify developmental mutations in the context of a dominant screen. We chose to implement a pharmacological sensitized dominant screen, as opposed to a recessive or genetic sensitized screen, which can be labor-intensive and costly due to breeding. 5FU was chosen as the sensitizing agent because it has a high potential to uncover mutations affecting multiple hematopoietic lineages and specifically targets progenitors, therefore, challenging the developmental pathways of the hematopoietic system.

Characterizing the effects of 5FU as a hematopoietic stress agent

The standard dose of 5FU (150 $\mu\text{g/g}$) can cause lethality, thus we wanted to understand how a reduced 5FU dose would affect the robustness of cytopenia induction. We demonstrated that a lower 5FU dose (100 $\mu\text{g/g}$) did not cause mortality in wild-type mice, and maintained a classic PLT recovery pattern, but substantially reduced the severity of anemia. A 5FU dose of 120 mg/g eliminated mortality in wild-type mice and maintained a robust induction of cytopenia and PLT overshoot.

Our study illustrates stark differences in 5FU response among commonly utilized laboratory strains of mice, as no two strains had identical recovery kinetics. The greatest concordance was observed between *BALB/cJ* and *C57BL/6J* strains, with no significant differences detected in the RBC and PLT recovery curves. The *129S1/ImJ* strain was similar to *C57BL/6J* and *BALB/cJ* strains; however, anemia was less severe and recovery from anemia and thrombocytosis was more rapid in *129S1/ImJ* mice. The recovery kinetics of the *C3H/HeJ* strain were strikingly different, specifically the RBC and WBC nadir occurred earlier and the cytopenia was less pronounced. These observations are likely caused by differences in the size of progenitor pools [17], the number of progenitors in cycle, or the expression of regulators in the cellular pathways that respond to hematopoietic stress. In addition, 5FU may be absorbed, metabolized, or excreted at different rates in each strain.

Platelets have a unique 5FU recovery pattern because they substantially overshoot their baseline values [9]. A reduction in megakaryocyte ploidy occurred as early as 18 hours after 5FU treatment and persisted until D4, indicating that a build-up of immature megakaryocytes and/or loss of mature megakaryocytes occurred. Within 1 week of 5FU treatment, a multifold increase in total megakaryocytes was observed, which correlated with recovery from thrombocytopenia. At 2 weeks the platelet overshoot was still present, although megakaryocyte numbers and ploidy had returned to baseline, suggesting that the platelets would return to normal levels shortly thereafter. Previously, it has been shown that 5FU treatment results in a decrease and subsequent rebound in CFU-megakaryocyte number, which parallels the drop in PLT numbers and subsequent PLT overshoot [11]. Thus, the prolonged PLT overshoot observed after 5FU treatment can be explained by the elevated CFU-megakaryocyte numbers.

RBC counts decreased profoundly after 5FU treatment, and recovery was substantially delayed compared to both PLT and WBC parameters, despite the rapid recovery of BFU-E and CFU-E in the BM. A sharp increase in nonerythroid splenic progenitors and immature RBC in the peripheral blood correlates with RBC recovery. Bone morphogenetic protein 4 signaling is critical in recovering from treatment with phenylhydrazine, a drug that induces stress erythropoiesis and mobilizes a specialized population of stress erythroid progenitors in the spleen [18,19]. Although 5FU and phenylhydrazine both induce stress erythropoiesis, PHZ causes an expansion of splenic BFU-E (red colonies) and alters the recovery kinetics in mice defective for bone morphogenetic protein 4 signaling, neither of which occur in mice treated with 5FU (R.F.P., unpublished data). These differences may be because phenylhydrazine destroys erythrocytes to induce anemia, while 5FU affects progenitor cells, thereby blocking production of new erythrocytes.

Use of 5FU in an ENU dominant screen

The *129S1/ImJ* and *C57BL/6J* strain combination was used for the dominant screen because we had already optimized the ENU dose on the *129S1/ImJ* strain, the *129S1/ImJ* × *C57BL/6J* (129:B6) strain combination was already in use in our dominant screen, and both mouse strains had similar RBC and PLT recovery kinetics. For a 5FU screen to be successful, a thorough understanding of the recovery kinetics of the 129:B6 strain combination was necessary to minimize detection of false-positive G1 mice. Therefore, we used the 129:B6 hybrid strain to construct 5FU recovery curves for RBC, PLT, and WBC, which were then used to select optimal testing days for analysis of cytopenia recovery. A baseline value was collected on D0, which was critical for determining whether the G1 animals had a pre-existing hematological defect that contributed to the 5FU phenotype. As PLT recovery occurred quickly, PLT nadir was analyzed on D6, recovery on D8, and potential PLT overshoot on D13. RBC and WBC recovery was assessed on D8 and D13.

A post-screen analysis was performed on all G1 males to determine the effectiveness of the selected testing days to assess RBC, PLT, and WBC recovery. The platelet recovery pattern of G1 mice was very consistent. The testing days chosen to assess delayed PLT recovery are highly effective, with few false positives, as only 0.5% of G1 mice were outliers. RBC recovery was not well synchronized in G1 mice, suggesting that the lower 5FU dose was not efficient at inducing anemia. As predicted by control data, WBC recovery of G1 animals was variable.

The similarity in PLT recovery between the *129S1/ImJ* and *C57BL/6J* mouse strains made PLT recovery an ideal phenotype to monitor in this sensitized ENU screen, which was confirmed in the post-screen analysis. Delayed PLT recovery and PLT overshoot were the two classes of PLT phenotypes detected in the sensitized screen. We identified three delayed PLT recovery outliers and 10 PLT overshoot outliers. Two phenodeviants were selected for further analysis: high PLT but normal PLT recovery (*7323*) and increased PLT overshoot on D13 (*7325*).

The *7323* strain showed high PLT baseline values but had no detectable differences in PLT recovery following 5FU treatment. The high baseline PLT phenotype was heritable at low penetrance and mapped to the distal end of chromosome 11 (10 Mb region comprising 152 genes). The high PLT phenotype was seen predominantly in males, but did not show linkage to the X chromosome.

The *7325* strain was an outlier for increased PLT overshoot on D13. Nine other G1 mice presented with a similar phenotype. In *7325* mice, this trait was heritable with low penetrance and mapped to the distal 45-Mb region on chromosome 11. Although low penetrance was observed in this line, the false-positive rate was minimal. Mapping in subsequent generations revealed that the defect in strain *7325* maps to an 8.84-Mb region on chromosome 11, consisting of 100 genes. We have recently utilized massive parallel sequencing to identify a point mutation in an ENU allele (Anderson et al., unpublished data). The mutation(s) underlying these phenotypes will be identified using a similar strategy. Because the mechanisms for PLT overshoot are not well understood, the causative mutations for strain *7325* may provide important insight into 5FU-induced PLT recovery kinetics (and possibly recovery from other chemotherapeutics) and megakaryopoiesis.

In an alternative approach, Hilton, Alexander and colleagues have performed a genetic modifier screen to identify dominant mutations that would suppress thrombocytopenia phenotype in *mpl^{-/-}* mice [20]. Four novel mutations were identified, which reinforced the importance of c-Myb [20] and p300 [21] in megakaryopoiesis, as well as identified a role for Suz12 in hematopoietic stem cells and megakaryopoiesis [22]. This approach demonstrates the power of utilizing genetic sensitization to identify dominant ENU mutations that cause defects in the megakaryocyte and platelet lineage. Our 5FU screen uses a pharmaceutical sensitization approach to identify dominant mutations in megakaryopoiesis/thrombopoiesis and stress thrombopoiesis.

A recessive screen was performed by Papathanasiou and colleagues to examine embryonic hematopoiesis [17] by using flow cytometry to examine hematopoietic progenitors. Prior optimization experiments included analysis of lineage differences across eight strains of mice, and proved critical substantial differences were documented in progenitor frequency and detection among the strains. Like our study, this study also underscores the requirement of establishing precise baseline measurements to minimize false positives and maximize the ability to isolate mutant mice with neomorphic phenotypes.

Conclusions

We have illustrated that there are profound differences in 5FU response among four commonly utilized laboratory mouse strains. We have shown that *129/SvImJ* × *C57BL/6J* is an ideal strain combination for isolation of mutant mice with defects in the megakaryocyte lineage. Utilizing a sensitized screen based on 5FU recovery dynamics, we identified 14 ENU-generated mutants with altered PLT recovery and mapped the mutations of two strains to two distinct regions on chromosome 11. We have refined the mutation interval for strain *7325* to an 8.84-Mb region on chromosome 11. The mapping interval for strain *7325* encodes 100 genes, none of which are known to be regulators of thrombopoiesis or megakaryopoiesis, which provides strong evidence that our unique pharmacological sensitized screen can identify novel genetic determinants of the megakaryocyte/platelet lineage.

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Figure 1.

Analysis of dose response and strain-specific effects of 5FU. Each group of mice consists of two subgroups of 8 to 10 animals, one subgroup was tested at D0, 4, 8, 12, 16, and the second was tested at D0, 2, 6, 10, 14. The two subgroups were combined to create one group represented by a single recovery curve, which includes the baseline and data sampled every 2 days post-treatment. **(A)** 5FU dose response. *C57BL/6J* females were injected intraperitoneally with 5FU (100, 120, or 150 µg/g) and the recovery curves for RBC, PLT, and WBC were determined (left, center, and right, respectively). The degree of anemia was assessed for each dose by comparing the RBC at the nadir for each recovery curve (RBC nadir *t*-test: 120 vs 150, $p = 0.412$ NS; 120 or 150 vs 100, $p < 0.0005$). The 5FU dose of 100 and 120 µg/g induced similar levels of thrombocytopenia (PLT nadir *t*-test: 100 D6 vs 120 D6: $p = 0.16$) and PLT overshoot (PLT peak of overshoot: 100 D10 vs 120 D12; $p = 0.75$). The dose of 150 mg/g 5FU induced a unique PLT recovery curve compared to 100 µg/g and 120 µg/g (two-way repeat measures ANOVA: 120 vs 150; $p < 0.0001$; 100 vs 150; $p = 0.0001$). The overall WBC recovery kinetics were different in mice treated with 150 µg/g 5FU (two-way repeat measures ANOVA: 100 vs 120; $p = 0.656$; 100 vs 150 and 120 vs 150; $p < 0.005$). The WBC overshoot was higher for mice treated with 150 µg/g for each (peak WBC values *t*-test: 100 D10 vs 120 D12; $p = 0.559$; 150 vs 100 or 120; $p < 0.005$). **(B)** Effect of mouse strain on 5FU-induced cytopenia. *C57BL/6J*, *BALB/cJ*, *129S1/SVImJ*, and *C3H/HeJ* female mice were used for this comparison. The RBC recovery was compared between the four strains at baseline (one-way ANOVA: $p < 0.0001$), and *C3H/HeJ* was lower than other strains tested (*t*-test: *C3H/HeJ* vs *C57BL/6J*, *BALB/cJ*, or *129S1/SVImJ*; $p < 0.05$); and the degree of anemia (RBC nadir: ANOVA, $p < 0.0001$). The PLT recovery kinetics between the four mouse strains were analyzed for the following components: baseline (ANOVA, $p < 0.002$); induction of thrombocytopenia or PLT overshoot (one-way repeated measures ANOVA and Dunnett's post-test, $p < 0.05$: *C57BL/6J* D8-14; *BALB/cJ* D10-14; *129S1/SVImJ* D4-6, D10-14; *C3H/HeJ* D4, D8-14); and comparing overall PLT recovery kinetics (two-way repeated measures ANOVA, $p < 0.05$: *C3H/HeJ* vs *129S1/SVImJ* or *C57BL/6J* and *C57BL/6J* vs *129S1/SVImJ*). The ability of 5FU to induce leukopenia or WBC overshoot in the four strains was analyzed (one-way repeat measures ANOVA with Dunnett's post-test, $p < 0.05$: *C57BL/6J* D6-10; *BALB/cJ* D4-8 D14; *129S1/SVImJ* D4-8, *C3H/HeJ* D2-4 D8). The differences in WBC recovery between the mouse strains were

observed at baseline (one-way ANOVA, $p < 0.03$) and the height of WBC overshoot (one-way ANOVA, $p < 0.05$).

Figure 2.

Time course examining the alterations in the KTLS population and profile post-cytopenia induction. Each experimental cohort is represented by two groups of three *C57BL/6J* female mice treated at 5 weeks of age, one group was treated with phosphate-buffered saline (PBS) only and one with 5FU. Two to three cohorts were examined at 18 hours, 7 days, or 14 days after treatment. At each time point, BM cells were analyzed by flow cytometry for KTLS expression (c-kit⁺, Thy1^{lo}, Lin⁻, Sca-1⁺). All animals were analyzed individually. Each flow plot shows a Lin⁻Thy1^{lo} population on a c-kit and Sca-1 plot and the red box represents the KTLS gate, while the proportion of cells inside each box is shown as a percentage of total cell number and represents the average for a group in an individual experiment. The c-kit^{hi} KTLS-positive population is shown in the green box, percentage of this population shown in right corner. 5FU-treated mice, 18 hours post-treatment, showed a loss of c-kit^{hi}-expressing cells. A statistically significant difference was observed between PBS only and 5FU-treated groups in the c-kit^{hi} (*t*-test $p < 0.05$ in each cohort). The KTLS population increased in the 5FU-treated group on D7 (*t*-test $0.1 < p < 0.004$ in two cohorts).

Figure 3.

The effect of 5FU on BM and splenic-derived hematopoietic progenitors. Each experimental cohort was represented by two groups of three *C57BL/6J* female mice treated at 5 weeks of age, and each group was treated with either phosphate-buffered saline or 5FU. Three cohorts were examined at each time point of 18 hours, 7 days, and 14 days post-treatment. Data plotted represents the average $\pm$ standard error of mean for all three cohorts, representing a total of nine animals for each treatment at each time point. Statistically significant differences ($p < 0.05$) from baseline (D0) are indicated by a line over each group and an asterisk. **(A)** BM cellularity was assessed by the total number of cells isolated per femur flushed with Iscove's modified Dulbecco's medium + fetal bovine serum. **(B)** CFU-C frequency is shown as the number of CFU-C colonies per 2×10^4 BM cells. Each bar indicates total CFU-C number along with the frequency of CFU-granulocyte (CFU-G), CFU-macrophage (CFU-M), CFU-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM), CFU-granulocyte macrophage (CFU-GM), and BFU-E colonies. **(C)** Number of CFU-C per femur was calculated from (CFU-C freq/cell plated) $\times$ total BM cells per femur. **(D)** The frequency of CFU-E colonies per 2×10^4 BM cells. **(E)** Splenic CFU-C were calculated as the number of CFU-C colonies per 1×10^5 spleen cells and each bar shows both red colonies in black (BFU-E) and non-red colonies in white (all other CFU-C colony types: CFU-G, CFU-M, CFU-GEMM, CFU-GM).

Figure 4.

Megakaryocyte ploidy analysis. An experimental cohort is represented by two groups of three *C57BL/6J* female mice. Each group was treated at 5 weeks old with 5FU or phosphate-buffered saline (PBS). One experimental cohort was used for each time point of 18 hours, 4 days, 7 days and 14 days post-treatment. Data in each fluorescence-activated cell sorting plot is representative from one animal from each group. Megakaryocytes were identified by positive CD41 expression and the DNA content (ploidy) of each megakaryocyte was measured by propidium iodide (PI) intensity. PI intensity doubling was set at 2N, 4N, 8N, 16N, 32N, and 64N on CD41⁺ cells. Table 1 displays the average and standard deviation for each group as well as statistical analysis. Cells positive for CD41 expression were divided into two categories: region one (R1) DNA content of 2N, 4N, or 8N and region two (R2) DNA content of 16N, 32N, and 64N. (A) Ploidy analysis of CD41⁺ cell at 18 hours, 4 days, 7 days, and 14 days after 5FU or PBS treatment. (B) The percentage of CD41-expressing cells in the BM for control or 5FU group 18 hours, 4, 7, and 14 days animals after treatment. Statistically significant differences ($p < 0.05$) from baseline (D0) are indicated by a line over each group and an asterisk.

Figure 5.

ENU breeding scheme and selection of optimized 5FU testing days. **(A)** Our dominant ENU breeding scheme used for the 5FU screen. The mutagenized mouse strain was *129S1/SVImJ* and the backcross strain was *C57BL/6J*. **(B)** The recovery kinetics for G1 hybrid (B6:129) control males of RBC, PLT, and WBC are shown in blue. Four independent groups of F1 control mice were used to create a 5FU recovery curve (Group1: D0, D1, D5, D9; Group 2: D0, D2, D6, D8, D12, D16, D20; Group 3: D0, D3, D7, D11; Group 4: D0, D4, D8, D12, D16, D20). The testing days chosen for the 5FU screen to identify defects in multiple lineages in G1 hybrid males are indicated by the black squares.

Figure 6.

Analysis of G1 for defects in PLT recovery after 5FU administration. (A) The RBC, PLT, and WBC recovery is shown for all G1 males tested in the 5FU screen. The horizontal red line in each graph indicates no change occurred, and points above the line indicate that the values were increasing between two time points, while points below the line indicate that values were decreasing between the two time points. Platelet overshoot was analyzed on D13 and all G1 mice tested are displayed in the graphs in (B) (females) and (C) (males). Values above the black line in panels (B) and (C) represent outliers (z-score > 2.75). The value for each G1 is expressed as a black dot, except outliers that are shown in color. Delayed PLT recovery was assessed on D6 and D8, where PLT values are decreasing or negative and all G1 mice were displayed in the graphs in panel (D) (females) and (E) (males). Values below the black line in panel (D) and (E) represent outliers or PLT values are decreasing between D6 and D8.

Figure 7.

Heritability assessment of PLT recovery mutants. In each graph, the black line represents the average of control mice and the error bars represents 1 SD. Outlier mice are shown in red. (A) The G1 founder for the 7321 line, presented with increased PLT overshoot. The 7321 line is heritable, with 15% penetrance. The graph only displays G2 males. (B) The G1 for the 7324 phenotype originally presented with delayed PLT recovery. The 7324 line is heritable with 20% penetrance. Only G2 females are displayed in graph. (C) The 7323 phenotype displays high platelets on D0, but normal PLT recovery. 7323 is heritable with a 30% penetrance. Only G2 males are shown. (D) 7325 phenotype has PLT overshoot on D13. 7325 is heritable with 5/54 female mice affected (18.5% penetrance).

Table 1

Megakaryocyte ploidy analysis after 5-FU administration

Time	Condition	2N	4N	8N	16N	32N	64N
18 Hours	Control	13.12 ± 0.94	11.53 ± 0.36	16.08 ± 1.17	47.78 ± 3.30	9.28 ± 2.13	0.24 ± 0.15
	5FU	18.42 ± 0.56**	14.86 ± 1.19*	19.03 ± 6.42	36.06 ± 2.12*	10.06 ± 6.07	0.10 ± 0.14
D4	Control	7.56 ± 1.90	4.67 ± 1.47	13.67 ± 1.51	60.21 ± 4.40	11.70 ± 1.49	0.93 ± 0.45
	5FU	5.23 ± 1.21	16.80 ± 0.05**	43.90 ± 0.49**	26.31 ± 0.60**	5.04 ± 0.49	1.26 ± 0.10
D7	Control	10.23 ± 1.88	7.43 ± 0.65	15.38 ± 2.35	53.14 ± 5.42	11.82 ± 1.79	1.04 ± 0.34
	5FU	5.40 ± 0.78*	5.48 ± 0.71*	18.98 ± 3.57	49.99 ± 2.58	17.72 ± 3.70	1.30 ± 0.73
D14	Control	8.92 ± 3.33	5.94 ± 2.48	12.61 ± 3.82	57.54 ± 5.88	12.94 ± 4.25	2.45 ± 1.12
	5FU	8.40 ± 2.00	6.97 ± 1.36*	25.29 ± 8.29	46.70 ± 2.79	9.16 ± 3.99	1.33 ± 0.17

* $p < 0.05$.** $p < 0.01$.

Table 2

Summary of 5FU G1 platelet screen

Condition	Total	Bred	Heritable	Mapped
G1 screened	546			
Increased PLT on D0 only	1	1	1	1
PLT delayed recovery	3	1	1	0
PLT overshoot	11	2	2	1
Total outliers	15	4	4	2