Conditional Deletion of Jak2 Reveals an Essential Role in Hematopoiesis throughout Mouse Ontogeny: Implications for Jak2 Inhibition in Humans

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Abstract

Germline deletion of Jak2 in mice results in embryonic lethality at E12.5 due to impaired hematopoiesis. However, the role that Jak2 might play in late gestation and postnatal life is unknown. To understand this, we utilized a conditional knockout approach that allowed for the deletion of Jak2 at various stages of prenatal and postnatal life. Specifically, Jak2 was deleted beginning at either mid/late gestation (E12.5), at postnatal day 4 (PN4), or at ~2 months of age. Deletion of Jak2 beginning at E12.5 resulted in embryonic death characterized by a lack of hematopoiesis. Deletion beginning at PN4 was also lethal due to a lack of erythropoiesis. Deletion of Jak2 in young adults was characterized by blood cytopenias, abnormal erythrocyte morphology, decreased marrow hematopoietic potential, and splenic atrophy. However, death was observed in only 20% of the mutants. Further analysis of these mice suggested that the increased survivability was due to an incomplete deletion of Jak2 and subsequent re-population of Jak2 expressing cells, as conditional deletion in mice having one floxed Jak2 allele and one null allele resulted in a more severe phenotype and subsequent death of all animals. We found that the deletion of Jak2 in the young adults had a differential effect on hematopoietic lineages; specifically, conditional Jak2 deletion in young adults severely impaired erythropoiesis and thrombopoiesis, modestly affected granulopoiesis and monocytopenia, and had no effect on lymphopoiesis. Interestingly, while the hematopoietic organs of these mutant animals were severely affected by the deletion of Jak2, we found that the hearts, kidneys, lungs, and brains of these same mice were histologically normal. From this, we conclude that Jak2 plays an essential and non-redundant role in hematopoiesis during both prenatal and postnatal life and this has direct implications regarding the inhibition of Jak2 in humans.

Introduction

Hematopoiesis is the process whereby hematopoietic stem cells in the bone marrow give rise to the terminally differentiated cells in the peripheral blood. The process is exquisitely controlled by a number of cytokines including granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), erythropoietin (EPO), and thrombopoietin (TPO) to name a few. The binding of these cytokines to their cognate receptors on hematopoietic cells results in the activation of at least ten different Src family kinases and all four Janus family kinases within these cell types [1–4]. These activated kinases then phosphorylate a number of different intracellular substrates resulting in appropriate cell proliferation, differentiation, and subsequent hematopoiesis.

Janus kinase 2 (Jak2) is a member of the Janus family of tyrosine kinases. It was cloned in 1992 and found to be ubiquitously expressed in a number of animal tissues including hematopoietic organs [5]. Early signaling studies found Jak2 to be a critical mediator of both growth hormone and erythropoietin-dependent signaling [6,7]. The in vivo importance of Jak2 in cytokine-dependent signaling was confirmed several years later when germline deletion of Jak2 in mice resulted in embryonic lethality by day 12.5 (E12.5) due to a lack of hematopoiesis [8,9]. Despite the large number of kinases that are activated during hematopoiesis, these results indicated that at least during early embryonic development, there is no redundancy for the functional loss of Jak2. However, what role, if any, that Jak2 might play in hematopoiesis during the later stages of embryonic development, as well as in postnatal life, has not been previously explored.

In 2005, several groups independently reported a valine to phenylalanine substitution mutation at amino acid 617 of Jak2, in a large percentage of myeloproliferative neoplasm (MPN) patients...
Jak2

for 30 seconds, 60°C for 40 seconds, and 72°C for 40 seconds. Reaction products were separated and visualized on 2% agarose gels.

Histologic Analysis

Mouse livers, spleens, kidneys, brains, hearts, and femurs were fixed overnight in buffered formalin and femurs were decalcified for 16 hours. Tissues were subsequently dehydrated through graded alcohols, paraffin embedded, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). Sections were examined under a light microscope for cellularity, necrosis, fibrosis, hemorrhage, and other lesions.

Analysis of Peripheral Blood Samples

Mouse peripheral blood samples were obtained via submandibular bleeding. Complete blood counts (CBC) were determined by a veterinary pathologist using peripheral blood films that were prepared in parallel and stained with Dip Quick (Jorgensen Laboratories). For the longitudinal studies, a small drop (~5 μl) of blood was also applied to a HemoPoint H2 analyzer (Stabio Life Sciences) to measure hemoglobin concentrations.

Isolation of Bone Marrow

Bone marrow cells were prepared as previously described [24]. Briefly, femurs were flushed with 2 mL Dulbecco’s modified minimum essential medium using a 27-gauge needle. Single cell suspensions were then prepared by repeatedly passing the harvested bone marrow cells through the needle. After lysis of red blood cells, bone marrow cells were either immediately frozen in liquid nitrogen and stored at −80°C for subsequent analysis or used directly for flow cytometry as described below.

Real-time PCR

RNA was isolated from liver and bone marrow cells using the RNeasy Mini Kit (QIAGEN) and DNA contamination was removed using RNase-free DNase. cDNA was synthesized from 2 μg mRNA using the high capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed on cDNA templates with a Multicolor Real-Time PCR Detection System using TaqMan gene expression assays (Applied Biosystems). PCR amplifications were performed in triplicate for Jak2 (Mm00434577_m1), GBP-2 (Mm00494576_g1), and IRF-1 (Mm01288580_m1) along with parallel measurements of β-actin cDNA as an internal control.

Flow Cytometry of HSC Progenitors

Bone marrow was harvested and single cell suspensions were prepared. Cell surface markers were adopted from previous publications which were defined and optimized for flow cytometry [25,26]. Cells were biontin-labeled using a mouse hematopoietic lineage (Lin) flow panel (eBioscience #88-7774) and secondary labeled with Streptavidin-PE-Cy7 (eBioscience #25–4317). Specific fluorochrome-conjugated markers were APC anti-mouse CD117 (c-Kit, eBioscience #17–1171), Alexa Fluor anti-mouse Ly-6A/E (Sca-1, eBioscience #56–5981), eFluor 450 anti-mouse CD34 (eBioscience #48–0341), and Flk2-PE (BD #553842). Lineages were defined as long term hematopoietic stem cell (LT-HSC, Kit+; Sca1+; Linn1; Flk2-; CD34+), short term hematopoietic stem cell (ST-HSC, Kit+; Sca1+; Lin-; Flk2-; CD34+), multipotent progenitor (MPP, Kit+; Sca1+; Lin-; Flk2-; CD34+), LSK (c-Kit+; Sca1+; Lin-; stem cell.
enriched population), common myeloid progenitor (CMP, Kit<sup>+</sup>; Sca<sup>-</sup>; Lin<sup>-</sup>; Flk2<sup>+</sup>; CD34<sup>+</sup>), common lymphoid progenitor (CLP, Kit<sup>low</sup>; Scalow; Lin<sup>-</sup>; Flk2<sup>+</sup>; CD34<sup>+</sup>), megakaryocyte-erythrocyte progenitor (MEP, Kit<sup>+</sup>; Sca<sup>-</sup>; Lin<sup>low</sup>; Flk2<sup>+</sup>; CD34<sup>-</sup>), and granulocyte-macrophage progenitor (GMP, Kit<sup>+</sup>; Sca<sup>-</sup>; Lin<sup>low</sup>; Flk2<sup>+</sup>; CD34<sup>+</sup>).

Flow Cytometry of Splenic Lymphocytes

Single-cell suspensions were prepared from spleens using 70 μm sieves and erythrocytes were lysed. Cells were labeled with anti-CD45 APC (BD #559864), anti-B220 PE (BD #561878), and anti-CD3ε FITC (BD #557666). Populations were defined as either T lymphocytes (CD45<sup>+</sup>; CD3<sup>+</sup>) or B lymphocytes (CD45<sup>+</sup>; B220<sup>+</sup>) using FACS Diva Software (3.0).

Colonies Forming Assays

For CFU-GEMM, CFU-GM, and BFU-E analysis, 2 × 10<sup>4</sup> bone marrow cells were seeded in 35 mm dishes using methylcellulose-containing medium (STEMCELL Technologies) and colonies were scored 7–9 days later. For CFU-Meg analysis, 2 × 10<sup>4</sup> bone marrow cells were seeded in 35 mm dishes using MegaCult-C medium (STEMCELL Technologies). After 8 days in culture, the semi-solid medium was dehydrated, fixed with acetone, and evaluated using acetylcholinesterase staining.

Statistical Analysis

All results were expressed as means ± SEM. Statistical comparison of the different conditions was performed either by a pair wise t test or by an unpaired Student’s t-test. *, p<0.05; **, p<0.01.

Figure 1. Deletion of Jak2 during mid/late gestation results in impaired erythropoiesis. (A) Cartoon illustrating the times at which Jak2 was deleted from mice via Tamoxifen injection and the times at which necropsy was performed. The three selected times for Jak2 deletion are mid/late gestation (E12.5), early prenatal (PN4), and early adult (PN35/PN63). Also shown are the times when necropsy was performed on each group; E16.5–E17.5, PN17–PN20, and PN91, respectively. (B) Representative control and Jak2 cKO E16.5 yolk sacs are shown as well as the PCR analysis that confirmed both the presence of Cre and the null allele in the Jak2 cKO embryos (n = 8), but not the controls (n = 12). Size bars = 2 mm. (C) E17.5 embryos connected to visceral yolk sacs (top). Blood-filled umbilical vessels were seen in the control embryos, but not the Jak2 cKO embryos (bottom). Size bars = 2 mm. (D) When compared to controls, spleens from the Jak2 cKO mice were paler (top) with visible histopathological necrosis (bottom, indicated by arrows). Size bars = 2 mm while lower panel size bars = 10 μm. (E) While livers from the control embryos appeared normal, livers from the Jak2 cKO embryos were similarly smaller and paler (top) and histopathology of E17.5 embryonic livers showed marked hypocellularity and reduced erythropoiesis in the Jak2 cKO mice (bottom). Upper panel size bar = 2 mm while lower panel size bars = 10 μm. (F) Determination of Jak2 mRNA levels in the liver from both genotypes (n = 3 for each genotype). **, p<0.01.

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Results

Tamoxifen-inducible Deletion of Jak2 during Mid/late Gestation Results in Hematopoietic Insufficiency and Death

Previous studies demonstrated that germline deletion of Jak2 results in embryonic lethality at E12.5 due to impaired hematopoiesis [8,9]. The floxed Jak2 mice that we obtained for this current study were created independently of those previous two works. Specifically, the floxed Jak2 mice used here had loxP sites introduced around the ATG start codon in exon 2 [21]. Although it was shown that germline deletion of Jak2 in our mouse [21] recapitulates the previous two reports [8,9], we first wanted to demonstrate that germline deletion of Jak2 in our hands would yield a similar result. We found that germline deletion of Jak2 derived from the Jak2 cKO mice in our hands resulted in a phenotype that was identical to the previous Jak2 conventional knockouts, as all our Jak2 null embryos were dead and partially resorbed at E13.5 and the reason for death was impaired hematopoiesis (Figure S1). As such, these results demonstrated a proof-of-concept in that they recapitulate the phenotype of the previously reported Jak2 conventional null embryos and therefore allowed for the deletion of Jak2 at later stages of prenatal and postnatal life.

To determine the role of Jak2 beyond germline deletion, we selected three different stages for Jak2 elimination; namely, mid/late gestation (E12.5), early postnatal life (PN4), or early adulthood (~2 months of age). Figure 1A provides a summary of these optimized injection times and the days at which necropsy was performed for each specific cohort.

For the mid/late gestational experiments, timed matings were set with ROSA26CreER/+;Jak2f/f males and ROSA26f/f females. Tamoxifen (TM) was then injected into pregnant dams at 12.5 days post coitum (dpc). No ROSA26CreER/+;Jak2f/f newborn pups were found from the first several litters, indicating gestational lethality. To determine why lethality occurred in the Jak2 cKO mice, embryos from pregnant dams injected with TM at 12.5 dpc were examined at E17.5. All ROSA26CreER/+;Jak2f/f embryos (n = 13) were non-viable and resorbed at E17.5 when compared to controls (n = 20). The Jak2 conditional mutants were easily distinguished by pale yolk sacs and undersized embryos (n = 13) were non-viable and resorbed at E17.5 when compared to controls (n = 18). Jak2 cKO mice administered TM at PN4 were clearly distinguishable from the control mice due to pallor and a reduced body size at PN17, and quantification of this size difference found it to be significant (Figure 2A). When compared to the controls, the tails, paws, and gastrointestinal system of the Jak2 cKO mice were very pale, suggestive of poor peripheral perfusion (Figure 2B). Analysis of the peripheral blood revealed significantly reduced numbers of platelets and marked microcytic hypochromic anemia and the hematocrits of the Jak2 cKO mice were reduced by ~85%, when compared to controls (Figure 2C).

Characterization of the hematopoietic potential of these animals continued with analysis of the bone marrow, spleen, and liver. The Jak2 cKO marrow was markedly hypocellular and quantification of these data indicated that this difference was significant (Figure 2D). We found that the Jak2 cKO mice displayed an 80% reduction in spleen weight to body weight ratios when compared to controls (Figure 2E). Gross and histologic examination found that the Jak2 cKO spleens were hypoplastic, exhibited disorganized red and white pulp, and displayed erythroid extramedullary hematopoiesis (Figure 2F). Interestingly, analysis of splenic lymphocytes found that the percentage of lymphocytes in the Jak2 cKO mice were significantly elevated, when compared to controls (Table 1). Histologic examination of the liver revealed that the Jak2 cKO mice exhibited hepatocellular atrophy and lobular collapse when compared to controls (Figure 2H).

Collectively, the data in Figure 2 and Table 1 indicate that loss of Jak2 beginning at PN4 results in animal death by PN25 via hematopoietic insufficiency and severe anemia.

Tamoxifen-inducible Deletion of Jak2 Beginning in Early Adulthood Results in Impaired Hematopoiesis, but Higher Survival Rates

We next wanted to determine what effect, if any, the loss of functional Jak2 would have on adult animals. To gain some sense of how best to optimize Jak2 deletion in early adults, we used Rosa26-LacZ reporter mice, injected them with TM, and then examined β-gal expression patterns. This was an important issue as the data in Figures 1 and 2 showed that while TM is highly effective at deleting Jak2 (ie, >99%), however, it is not absolute. We found that a total of six TM injections for adult mice, comprised of three consecutive days of TM injections starting at PN35 and three consecutive days of TM booster injections starting at day 63, provided the highest level of β-gal expression when tissues were examined at PN91 (data not shown).

To determine the consequence of Jak2 deletion during early adulthood, control (ROSA26CreER/+;Jak2f/f) and Jak2 cKO (ROSA26CreER/+;Jak2f/f) mice were injected with TM following the optimized injection paradigm. Overall, we found that adult stage deletion of Jak2 resulted in a gross phenotype that was noticeably milder than that observed with the mid/late gestational and early postnatal Jak2 deletions. This was supported by the fact that death was observed in only 20% of the adult Jak2 cKO mutants, when compared to controls (Figure 3A). To gain a better understanding of the hematopoietic system within these animals, tissues were
harvested at PN91 and analyzed. With respect to the peripheral blood, CBC and blood film evaluation indicated a number of abnormalities in the Jak2 cKO mice including significant reductions in red blood cells, hemoglobin, hematocrit, mean corpuscular hemoglobin, platelets, mean platelet volume, white blood cells, lymphocytes, monocytes, eosinophils, and basophils (Tables 2 and 3). The peripheral blood films from Jak2 cKO mice contained poikilocyte types and other morphologic changes including acanthocytes, schistocytes, echinocytes, elliptocytes, spheroechinocytes, stomatocytes, and hypochromic microcytic erythrocytes (Figure 3B). Hemoglobin crystals were also qualitatively increased in Jak2 cKO mice, when compared to controls.

The spleen weight to body weight ratios were reduced by an average of 46% in the Jak2 cKO mice when compared to the controls and this was significant (Figure 3C). Hematocrits plotted as a function of genotype indicating severe anemia in the Jak2 cKO mice. Size bars = 10 μm. (D) Representative bone marrow sections from both genotypes and quantification of marrow cellularity plotted as a function of genotype. Size bars = 500 μm for 4X images and 50 μm for 40X images. (E) Representative spleens from both genotypes and spleen weight to body weight ratios plotted as a function of genotype. Size bar = 5 mm. (F) Histologic sections through the spleen are shown which indicate a hypoplasia phenotype in the Jak2 cKO mice. Size bars = 500 μm for 4X images and 50 μm for 40X images. (G) Representative liver sections from both genotypes showing hepatocellular atrophy in the Jak2 cKO tissue, but not in the control tissue. Size bars = 50 μm for 40X images and 10 μm for 100X images. (H) Determination of Jak2 mRNA levels in the liver from both genotypes. **, p<0.01. doi:10.1371/journal.pone.0059675.g002

Jak2 is expressed in nearly every tissue in the body and it has been implicated in a number of other pathologies including renal injury, hypertension, and heart failure [20]. To determine what effect, if any, that deletion of Jak2 had on non-hematopoietic organs, we also examined heart, kidney, lung, and brain sections from these same TM treated animals (Figure 4). Overall, there was no marked difference in the histological appearance of these tissues between the two genotypes even though their hematopoietic systems were notably different.
The liver and bone marrow (Figure 5B). Jak2 cKO mice were virtually the same as those of controls in both
in the Jak2 cKO mice were reduced by
mice were euthanized at days 56 and 147 so that the levels of Jak2
hemoglobin levels returned to baseline levels by day 147. To
the nadirs occurring around days 56 and 98. Interestingly, the
and subsequent Jak2 deletion resulted in cyclic erythropoiesis with
mRNA levels in the liver and a 20% death rate. Lastly, there were
abnormal hematopoiesis characterized by reduction in marrow
ectric tissues. An alternate explanation could be that in adulthood,
remaining Jak2 expressing cells could re-populate the hematopoietic
tissues. If this were the case, then over time,
newly created Jak2 compound mutant (ROSA26 CreER/
(Tamoxifen-inducible Deletion of Jak2 Significantly
Role of Jak2 in Hematopoiesis throughout Ontogeny

Table 1. Analysis of splenic lymphocytes at PN15 mice.

| Absolute cell count per animal (x10⁶) | CD45+ B220+ | CD45+ CD3+ | CD45+ B220+ CD3+ | Total # of splenocytes |
|--------------------------------------|-------------|-------------|-------------------|------------------------|
| Control                              | 29.4 ± 1.3  | 8.9 ± 2.0   | 7.4 ± 1.1         | 142.3 ± 7.1            |
| cKO                                  | 10.4 ± 2.2  | 7.5 ± 1.7   | 5.4 ± 1.1         | 25.6 ± 1.2             |
| Number of events per 10⁶ total events |             |             |                   |                       |
| Control                              | 2016 ± 89   | 609 ± 139   | 506 ± 76          |                        |
| cKO                                  | 4072 ± 863  | 2943 ± 653  | 2099 ± 412        |                        |
| Significance p<0.01                  |             |             |                   |                       |

After TM injection at PN4, control (n = 3) and Jak2 cKO (n = 4) spleens were
harvested at PN15 and subjected to flow cytometry for B cell (CD45+, B220+)
and T cell (CD45+, CD3+) markers. Shown are the absolute numbers of cells per
animal. Given that the total number of splenocytes are significantly reduced in
the Jak2 cKO mice due to the smaller spleen sizes, the numbers of B cells and T
cells are also shown after normalization per 10⁶ events.

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Collectively, the data in Tables 2 and 3 as well as Figures 3 and 4 indicate that deletion of Jak2 in young adulthood results in
abnormal hematopoiesis characterized by reduction in marrow
cellularity, atrophied spleens, and reduced peripheral blood cell
counts. This was coincident with a ~88% reduction in Jak2
mRNA levels in the liver and a 20% death rate. Lastly, there were
no marked differences in the histological appearance of the hearts,
kidneys, lungs, and brains of these same TM treated animals.

The Higher Survival Rate of the Adult Jak2 cKO Mice is
due to Re-population of Hematopoietic Tissues with Jak2
Expressing Cells

As opposed to the 100% lethality observed in the mid/late
gestational and early postnatal deletions, deletion of Jak2 from
young adult mice resulted in only a 20% death rate. One
explanation for this was that TM was limited in its ability to delete
Jak2 from the tissues. If this were the case, then, in time,
surviving Jak2 expressing cells could re-populate the hematopoietic
tissues. An alternate explanation could be that in adulthood,
there is functional redundancy by other proteins for the loss of
Jak2 and this in turn allows for improved survival. To gain a better
understanding of this process, mice of both genotypes were again
subjected to the TM regimen at days PN35 and PN63. The
expression analysis. Relative to the control samples, we found that
both GBP-2 and IRF-1 were significantly reduced in Jak2 cKO
livers across all three time points, thereby confirming a functional
loss of signaling that is known to be downstream of Jak2 (Figure 6).
Furthermore, whereas the magnitude of the decreased expression
of IRF-1 in the Jak2 cKO mice was similar across all three time

The data in Figs. 5A and 5B show that the levels of hemoglobin in
the Jak2 cKO mice correlate positively with Jak2 expression
levels in the liver and marrow. As such, these data suggest that
the higher survival rate observed in adult animals is more likely due to
repopulation of Jak2 expressing cells in hematopoietic tissues,
rather than compensation by other proteins functioning in an
environment that is devoid of Jak2. To demonstrate this
experimentally, we created a compound mutant mouse that was
composed of one floxed and one null Jak2 allele (f/f/Δ). The advantage of this mouse is that one Jak2 allele in each cell has already been deleted and hence, Cre recombinase only needs to
delete the remaining Jak2 allele.

For this experiment, three genotypes were used; control
(ROSA26 wildtype), Jak2 cKO (ROS26 CreER/f/Jak2f/f) and
the newly created Jak2 compound mutant (ROS26 CreER/
f/Jak2f/f/Δ). Beginning on day 35, the mice received the TM
regimen and hemoglobin levels were again determined weekly.
Similar to Figure 5A, injection of TM into the control mice
(ROSA26 wildtype) had no effect on hemoglobin levels while TM
injection into the Jak2 cKO mice (ROS26 CreER/f/Jak2f/f)
once again caused a significant decrease in the hemoglobin levels with
the nadir occurring around day 56 and increasing by day 63
(Figure 5C). However, TM injection into the Jak2 compound
mutants (ROS26 CreER/f/Jak2f/f/Δ), resulted in an even greater and
more sustained decrease in the hemoglobin levels and 100% mortality
n = 9) by day 72.

When taken together, the data in Figure 5 demonstrate that
deletion of Jak2 in young adults temporarily reduces the
hemoglobin levels to 6–8 g/dL and this is associated with a 20% death rate. At day 91, these animals have a number of
hematopoietic abnormalities including peripheral blood cytope-
nias with abnormal erythrocyte morphology, decreased marrow
cellularity, and splenic atrophy. However, the incomplete deletion
of Jak2 by TM leaves some Jak2 expressing cells intact and the
subsequent repopulation of the hematopoietic tissues by these cells
normalizes the hemoglobin levels by day 147. When the f/Δ mice
were injected with TM, there was a pronounced and sustained
reduction (<5 g/dL for more than 7 days) of the hemoglobin
concentrations and ensuing death. From this, we conclude that
inmate Jak2 actively restores hematopoietic homeostasis in the
adult Jak2 cKO mouse, but that elimination of Jak2 via the
combined effect of the null allele and TM-induced deletion of the
floxed allele, results in death.

Tamoxifen-inducible Deletion of Jak2 Significantly
Attenuates GBP-2 and IRF-1 Expression at all three
Deletion Time Points

Conditional deletion of Jak2 beginning at either E12.5, PN4, or
PN35 results in marked hematopoietic defects characterized by
a lack of definitive hematopoiesis/erythropoiesis (Figures 1, 2, 3).
To demonstrate that these defects were consistent with a loss of
signaling that is downstream of Jak2, we conducted gene profile
analysis on two genes whose expression is known to be highly Jak2-
dependent; namely, GBP-2 and IRF-1 [8,9]. Here, Jak2 was
conditionally deleted from mice via TM injection starting at either
E12.5, PN4, or PN35 and livers were subsequently harvested at
E17.5, PN19, and PN56, respectively. mRNA was extracted from
the livers, reversed transcribed, and subjected to quantitative gene
expression analysis. Relative to the control samples, we found that
both GBP-2 and IRF-1 were significantly reduced in Jak2 cKO
livers across all three time points, thereby confirming a functional
loss of signaling that is known to be downstream of Jak2 (Figure 6).
Furthermore, whereas the magnitude of the decreased expression
of IRF-1 in the Jak2 cKO mice was similar across all three time
Figure 3. Tamoxifen-inducible deletion of Jak2 during early adulthood results in impaired hematopoiesis and lower mortality rates. (A) Kaplan-Meier survival curves for control (n = 17) and age-matched Jak2 cKO mice (n = 34). (B) Representative peripheral blood films from both genotypes showing the presence of anemia, thrombocytopenia, and poikilocytosis in the Jak2 cKO mice, but not the controls. Size bars = 10 μm. (C) Representative spleens from both genotypes and spleen weight to body weight ratios plotted as a function of control (n = 10) and Jak2 cKO (n = 10) genotypes. Histologic sections of spleen found an abnormal splenic architecture in the Jak2 cKO mice characterized by atrophied and disorganized white pulp. Size bars = 2 mm for top panel, 500 μm for 4X images and 50 μm for 40X images. (D) Representative bone marrow sections from both genotypes indicating increased adipose deposits in the Jak2 cKO mice, but not the controls. Also shown is bone marrow cellularity plotted as a function of control (n = 4) and Jak2 cKO (n = 5) genotypes. Size bars = 500 μm for 4X images and 50 μm for 40X images. (E) Representative liver sections showing diffuse centrilobular vacuolar degeneration in the Jak2 cKO tissue, but not in the control tissue. Size bars = 500 μm for 4X images, 50 μm for 40X images, and 10 μm for 100X images. (F) Determination of Jak2 mRNA levels from control (n = 4) and Jak2 cKO (n = 5) livers. **, p<0.01.

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Table 2. CBCs from adult mice at Day 91.

|       | RBC (M/L) | Hb (g/dL) | HCT (%) | PLT (K/μL) | MCV (fL) | MCH (pg) | MCHC (g/dL) | RDW (%) | MPV (fL) | PDW (%) |
|-------|-----------|-----------|---------|------------|-----------|----------|-------------|---------|----------|--------|
| Control | 9.39±1.10 | 10.14±1.10 | 44.20±4.34 | 1058±43 | 47.24±3.18 | 10.81±0.53 | 22.93±0.98 | 20.59±2.05 | 3.94±0.35 | 31.44±3.51 |
| cKO    | 5.86±1.43 | 5.67±1.14 | 25.50±4.26 | 292±21 | 44.47±5.24 | 9.79±0.60 | 22.12±1.45 | 18.20±2.73 | 3.40±0.39 | 29.54±3.62 |
| % of Control | 62.4% | 55.9% | 57.7% | 27.6% | 94.1% | 90.5% | 96.5% | 88.4% | 86.2% | 90.4% |
| Significance | p<0.05 | p<0.05 | p<0.05 | p<0.05 | NS | p<0.05 | NS | p<0.05 | NS | NS |

Shown are the absolute CBC values for control (n = 7) and Jak2 cKO (n = 7) mice and the values after they are normalized to control values. RBC (red blood cell), Hb (hemoglobin), HCT (hematocrit), PLT (platelet), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), RDW (red cell distribution width), MPV (mean platelet volume), and PDW (platelet distribution width). NS, not significant.

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points, the effect of Jak2 deletion on GBP-2 expression in these same mice was greatest in early post natal life and least during late embryogenesis.

In summary, the data in Figure 6 indicate that deletion of Jak2 impacts Jak2-dependent downstream signaling as determined by the reduced levels of GBP-2 and IRF-1 mRNA in the livers of Jak2 cKO mice, relative to controls. Furthermore, the effect of Jak2 deletion on IRF-1 expression was similar across the three time points whereas the effect of Jak2 deletion on GBP-2 varied with the age of the animal.

### Table 3. CBCs from adult mice at Day 91.

|        | WBC (K/µL) | Neutrophils (K/µL) | Lymphocytes (K/µL) (%) | Monocytes (K/µL) (%) | Eosinophils (K/µL) (%) | Basophils (K/µL) (%) |
|--------|-------------|--------------------|------------------------|---------------------|------------------------|----------------------|
| Control| 12.23±2.85  | 3.65±0.89          | 29.93±3.92             | 6.88±1.64           | 56.57±4.37             | 1.19±0.29            |
| cKO    | 6.76±3.59   | 2.98±2.34          | 40.02±11.70            | 3.23±1.21           | 52.86±11.87            | 0.45±0.24            |
| % of Control | p<0.05 | NS | NS | p<0.05 | p<0.05 | p<0.05 |
| Significance | p<0.05 | NS | NS | p<0.05 | p<0.05 | p<0.05 |

Shown are the absolute CBC values for control (n = 7) and Jak2 cKO (n = 7) mice and the values after they are normalized to control values. WBC (white blood cell). NS, not significant.

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Figure 4. Histology of non-hematopoietic organs. Tissues were taken from Control (A-H) or Jak2 cKO (I-P) mice. Shown are representative hearts (A, E, I, and M), kidneys (B, F, J, and N), lungs (C, G, K, and O), and brains (D, H, L, and P). The relative magnifications are 4x (D and L), 10x (A–C and I–K), and 40x (E–H and M–P). For A and I, the yellow arrows indicate equal wall thickness and RV = right ventricle. For C and K, Br = bronchus. Size bars = 500 µm (D&L), 100 µm (A–C, I–K), and 50 µm (E–H, M–P).

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Tamoxifen-inducible Deletion of Jak2 during Early Adulthood Significantly Diminishes Myeloid, but not Lymphoid Progenitors

While the role of Jak2 on hematopoietic progenitor populations has been well defined in early embryogenesis [8,9], the gestational lethality of Jak2 conventional knockout mice has precluded a similar examination in adult animals. Therefore, to determine the specific hematopoietic lineages that were impacted by Jak2 deletion in the adult mouse, we repeated the longitudinal study shown in Figure 5A whereby mice received the TM dosing regimen starting on day 35 and cohorts of mice were sacrificed either at baseline (Day 31), at the hematopoietic nadir (Day 56), or at recovery (Day 120). Within the spleen, we found no significant differences in the number of T cells and B cells at any of the three adult time points (Figure 7A). We then examined the numbers of hematopoietic progenitors within the bone marrow. Figure 7B displays representative flow cytometry plots at Day 56 while Figure 7C indicates the aggregate numbers of progenitors plotted as a function of both genotype and day. Overall, we found no significant decreases in any hematopoietic progenitors either at baseline (Day 31) or at recovery (Day 120). However, significant decreases were observed in the Jak2 cKO mice at the hematopoietic nadir (Day 56). Specifically, at Day 56, when compared to controls, the Jak2 cKO mice had significantly reduced numbers of LT-HSC, ST-HSC, MPP, LSK, CMP, MEP, and GMP, but not CLP.

To determine if the reduced numbers of stem and myeloid progenitors impacted the clonogenic growth potential of these cells, bone marrow cells were also plated in semi-solid media and the number of colony forming units were determined. We observed no significant differences between the two genotypes in the clonogenic growth potential for any of the hematopoietic progenitors collected either on Day 31 or on Day 120 (Figure 7D). However, at Day 56, Jak2 cKO had significantly reduced numbers of CFU-GEMM (3.0 ± 0.59 vs. 1.25 ± 0.2), CFU-GM (41.3 ± 3.0 vs. 24.3 ± 3.2), BFU-E (7.0 ± 1.8 vs. 0.8 ± 0.3), and CFU-MK (13.3 ± 1.6 vs. 2.0 ± 0.4), when compared to controls. Interestingly, although all myeloid lineages were significantly reduced in the Jak2 cKO cells when compared to the controls, there appeared to
be a differential effect of Jak2 on myelopoiesis as deletion of Jak2 severely impaired erythropoiesis and thrombopoiesis, but only had a mild to moderate effect on granulopoiesis and monocytopoiesis (Figure 7D).

Collectively, the data in Figure 7 indicate that deletion of Jak2 in the adult mouse diminishes myeloid, but not lymphoid progenitors. Furthermore, within the myeloid compartment, deletion of Jak2 in young adults severely impaired erythropoiesis and thrombopoiesis more so than granulopoiesis and monocytopoiesis. As such, Jak2 appears to have a specific yet critical effect on hematopoiesis in young adult animals.

Discussion

Hematopoiesis is a complex process involving scores of cytokines and dozens of kinases. At least ten different Src family kinases and all four Janus family kinases are expressed within hematopoietic cells [1–4]. In the case of Src family kinases, they are important for a number of hematopoietic responses including pro T cell and pro B cell development, megakaryocytepoiesis, thrombopoiesis, erythroblast expansion, and myeloproliferation [27–31]. In the case of Jak2, the germline deletion demonstrated the critical role of Jak2 in the establishment of the embryonic hematopoietic system [9,9]. However, the embryonic death of those mice precluded determination of the role of Jak2 in maintaining hematopoiesis in postnatal animals. Our results here demonstrate that Jak2 plays a similarly critical role in maintaining hematopoiesis in neonatal and adult animals. Further, the results show that there is no compensation by other kinases for the functional loss of Jak2 in neonatal and adult mice. As such, we conclude that Jak2 plays a critical and non-redundant role in hematopoiesis during prenatal and postnatal life.

Jak2 is ubiquitously expressed. Not surprisingly, it has been implicated in a number of pathologies including some of the heart, kidneys, lungs, and brain [32–35]. Analysis of these non-hematopoietic tissues from our adult mice found no marked gross or histological difference between the two genotypes. However, there were marked differences in the hematopoietic tissues that were harvested from these same animals. The implication of our results is that chronic Jak2 inhibition or absence of appreciable Jak2 function will impact the hematopoietic systems before these others.

We previously showed that adult mice that have only one functional Jak2 allele (ie, a 50% reduction in Jak2) are phenotypically normal [36]. Given that the Cre-mediated excision of Jak2 from the genome is an all or nothing event, in this current work, we used Jak2 mRNA levels as a surrogate for populations of cells. We found that an 88% reduction in Jak2 mRNA levels (ie, ~88% of cells experienced Cre-mediated Jak2 deletion) in the adult mice was associated with abnormal hematopoiesis characterized by reductions in marrow cellularity, atrophied spleens, reduced peripheral blood cell counts, and 20% mortality. In the adult Jak2 compound mutant mice where one allele was null and the other was deleted with TM (ROSA26 Cre/ER/+Jak2f/+), we found that there was a >95% decrease in Jak2 mRNA levels in the marrow and liver when compared to controls (ROSA26 Cre/+;Jak2f/+), and 100% mortality characterized by a severe lack of erythropoiesis. Thus, we have defined relative threshold levels of Jak2 that are needed to maintain hematopoietic viability within adult animals and described the associated phenotype that is observed when Jak2 function per se, is increasing lost (Figure 8).

Another important aspect of this work is the creation and characterization of the mice described herein. Specifically, the Jak2 cKO mice (ROSA26 Cre/ER/++;Jak2f/f) are a powerful tool for exploring the role of Jak2 in physiology and disease. In our case, we used the inducibility of Cre expression to escape the embryonic lethality of conventional Jak2 deletion and thereby defined specific roles for Jak2 in post natal life. Furthermore, primary cells can be obtained from the bone marrow or other tissues of these mice, cultured ex vivo, and Jak2 can then be deleted from the cells via the addition of TM to the culture media. This approach provides for a more precise inhibition of Jak2 when compared to Jak2 pharmacological inhibitors and unlike Jak2 siRNA, the down regulation/deletion of Jak2 within a given cell is permanent. That said, there are also limitations with these mice. For example, as noted earlier, a significant amount of time was spent identifying a TM injection protocol that would allow for the highest degree of Cre expression in all cells of an adult animal. Despite this optimization and subsequent 95% reductions of Jak2 mRNA levels in the bone marrow and liver, there was eventual re-population of hematopoietic tissues with Jak2 expressing cells. As such, the Jak2 compound mutant mice (ROSA26 Cre/ER/++;Jak2f/f) also described in this work should be used in combination with the cKO mice. Lastly, while our studies have defined novel roles for Jak2 in adult animals, they cannot distinguish between potential non-autono-
mous functions of Jak2. In other words, given that the deletion of Jak2 was from virtually every cell type in the mouse, it is possible that a supporting cell type (ie, stromal cell), may be providing the critical requirement for Jak2 function in hematopoiesis. However, given that the flow cytometry and clonogenic growth potential assays were done using individual hematopoietic cells, this is unlikely. That said, current studies which are utilizing allogenic bone marrow transplants between Jak2 cKO mice and littermate controls will help to resolve this issue.

Given the inability of first generation Jak2 inhibitors to provide marked bone marrow efficacy in the form of histopathologic, cytogenetic, or molecular remission [15–18], the overall impact of Jak2 inhibition on the bone marrow is not fully understood. In addition, it has recently been argued that more Jak2 inhibitors need to be developed and studied in order to not only identify more efficacious drugs, but to determine the consequence of long term Jak2 inhibition in animals [19,37]. In one regard, the TM-inducible system that we employed here is a case of extreme Jak2 inhibition. Rather than causing enzymatic inhibition of the protein, TM causes permanent deletion of Jak2 from genetically modified cells. Our results here demonstrate that virtual elimination of wild type Jak2 activity can ultimately lead to severe anemia/thrombocytopenia and even death. At the same time however, the ability of the few surviving Jak2 clones to subsequently re-populate hematopoietic tissues underscores the challenges of permanently ridding the bone marrow of targeted clones. Our results also appear to explain adverse events that were noted in a recent human study. Specifically, when the Jak2 inhibitor Ruxolitinib/Jakafi was administered to myelofibrosis patients with the intent of reducing blood transfusion dependency via the targeted suppression of mutant granulocyte/macrophage progenitors, it was reported that the drug was more likely to cause anemia and thrombocytopenia, than to correct them [18]. This is consistent with our observations whereby deletion of Jak2 in our

Figure 7. Tamoxifen-inducible deletion of Jak2 during early adulthood decreases stem cell and myeloid progenitors, but not lymphoid progenitors. (A) Spleens were harvested on the indicated days from the given genotypes and subjected to flow cytometry for analysis of T cell and B cell populations. Shown are the numbers of lymphocytes per 10^6 total events. 3–5 mice of each genotype were used on each day. (B) Representative flow cytometry plots for Control and Jak2 cKO genotypes indicating the markers and gating that was employed for these studies. (C) The numbers of hematopoietic progenitors plotted as a function of genotype and day. Deletion of Jak2 in young adults diminished the numbers of LT-HSC, ST-HSC, MPP, LSK, CMP, MEP, and GMP, but not CLP. (D) Bone marrow cells were isolated on the indicated days from the given genotypes and cultured in semi-solid medium. The numbers of colonies were then determined and plotted as a function of both genotype and day. Relative to controls, the numbers of CFU-GEMM, CFU-GM, BFU-E, and CFU-MK were all significantly reduced in the Jak2 cKO mice at day 56, but not at days 31 and 120. A total of 3–4 mice per genotype were used on each day and each sample was grown in duplicate. *, p<0.05. **, p<0.01. doi:10.1371/journal.pone.0059675.g007
adult animals virtually eliminated erythrocyte and thrombocyte growth potentials, but had only a moderate effect on granulocyte/macrophage potentials. As such, Jak2 inhibitors may be more suited for the treatment of polycythemia vera and/or essential thrombocythemia, diseases which are characterized by expanded erythrocyte and thrombocyte lineages, respectively. Given that there are clinical trials which are currently evaluating the use of Jak2 inhibitors for the treatment of these diseases, data will soon become available that will either support or refute this hypothesis. In another example of how the observations in our mice have therapeutic relevance, it was previously reported that the Jak2 inhibitor, SB1518, was effective at suppressing the growth of both myeloid and lymphoid malignancies [38]. Given our results here indicating normal lymphopoiesis in the Jak2 cKO mice, we conclude that the suppression of lymphoid malignancies by SB1518 is occurring via a mechanism that is independent of Jak2 inhibition. Consistent with this is the observation that SB1518 inhibits Tyk2 and FLT3 kinases with a potency that is similar to Jak2 [38] and thus, suppression of lymphoid malignancies by SB1518 may occur via the inhibition of one or both of these enzymes.

In summary, the loss of functional Jak2 at three different stages of mouse ontogeny results in hematopoietic insufficiency and death. From these results, we conclude that Jak2 plays a critical and non-redundant role in hematopoiesis during both prenatal and postnatal life. Furthermore, delineation of the hematopoietic lineages that are sensitive to the loss of Jak2 function in an adult animal has relevance to current attempts to inhibit Jak2 kinase function for the treatment of human diseases.

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