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Distinct *Brca1* mutations differentially reduce hematopoietic stem cell function

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Abstract

BRCA1 is a well-known DNA repair pathway component and a tissue-specific tumor suppressor. However, its role in hematopoiesis is uncertain. Here we report that a cohort of patients heterozygous for *BRCA1* mutations experienced more hematopoietic toxicity from chemotherapy than those with *BRCA2* mutations. To test whether this reflects a requirement for BRCA1 in hematopoiesis, we generated mice with *Brca1* mutations in hematopoietic cells. Mice homozygous for a null *Brca1* mutation in the embryonic hematopoietic system (*Vav1-iCre;Brca1*$^{F22–24/F22-24}$) developed hematopoietic defects in early adulthood that included reduced hematopoietic stem cells (HSCs). Although mice homozygous for a hu*BRCA1* knock-in allele (*Brca1*$^{BRCA1/BRCA1}$) were normal, mice with a mutant hu*BRCA1*/5382insC allele and a null allele (*Mx1-Cre;Brca1*$^{F22-24/5382insC}$), had severe hematopoietic defects marked by a complete loss of hematopoietic stem and progenitor cells. Our data show that *Brca1* is necessary for HSC maintenance and normal hematopoiesis, and that distinct mutations lead to different degrees of hematopoietic dysfunction.

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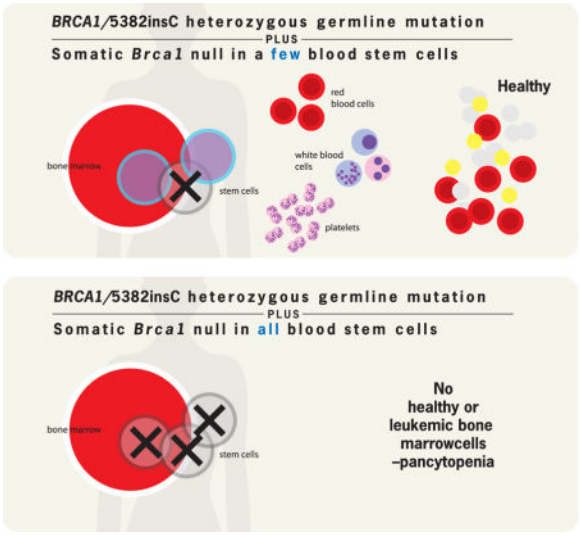
AUTHOR CONTRIBUTIONS

VEM and RAS designed experiments, collected data, interpreted results and edited the manuscript. RW, and TL collected, interpreted results and edited the manuscript. SJM interpreted data and edited the manuscript. TSR designed experiments, interpreted data and wrote the manuscript.

Mgbemena et al. report that hematopoietic stem cells have an absolute requirement for Brca1 to survive. They also show that humanization of the mouse *Brca1* gene with a knocked-in human *BRCA1* cDNA, but not a mutant *BRCA1*/5382insC cDNA, fully substitutes for mouse *Brca1* during both embryonic development and hematopoiesis.



INTRODUCTION

Hematopoietic stem cells (HSCs) depend upon DNA repair mechanisms to maintain their genomic integrity (Beerman et al., 2014; Mohrin et al., 2010; Rossi et al., 2007). Deficiencies in DNA repair proteins impair HSC function, although the nature and severity of the defects vary among DNA repair pathways. Deficiency for proteins involved in non-homologous end-joining does not affect HSC frequency or hematopoiesis in normal young adult mice but does reduce HSC function in response to stress (Rossi et al., 2007) and can lead to HSC depletion during aging (Nijnik et al., 2007). Deficiency for proteins involved in DNA mismatch repair does not appear to have major effects on hematopoiesis under normal conditions, but impairs the capacity of HSCs to reconstitute irradiated mice (Reese et al., 2003). Deficiency for homologous recombination-mediated double strand break repair proteins, however, can lead to hematopoietic failure in patients (Kottemann and Smogorzewska, 2013) and impair hematopoiesis in mice as well as impairing HSC function upon transplantation into irradiated mice (Bender et al., 2002; Carreau et al., 1999; Haneline et al., 1999; Ito et al., 2004; Navarro et al., 2006).

Fanconi anemia is caused by at least 18 different autosomal recessive mutants in the FA-BRCA repair pathway, including BRCA2 (Howlett et al., 2002; Xia et al., 2007), PALB2 (Reid et al., 2007), and BRIP1 (Seal et al., 2006). All three of these proteins physically interact with BRCA1 during DNA repair (Baer and Ludwig, 2002; Prakash et al., 2015; Xia et al., 2006; Zhang et al., 2009), raising the question of whether mutations in BRCA1 could also influence HSC function or hematopoiesis. Two individuals with developmental defects consistent with Fanconi anemia have been identified with genetic variants in both *BRCA1* alleles (Domchek et al., 2013; Sawyer et al., 2015); however, it is not clear that these were

all deleterious mutations and neither individual was reported to have hematopoietic defects. If loss-of-function mutations in *BRCA1* impair DNA repair in hematopoietic cells, this would have broad implications for patients with *BRCA1* mutations as these patients are at increased risk of certain cancers that are commonly treated with DNA-damaging chemotherapies.

Homozygosity for germline loss-of-function in *Brca1* is embryonic lethal in mice (Drost and Jonkers, 2009). Conditional deletion of *Brca1* from breast epithelium in mice leads to the development of breast cancer, but only when combined with p53 deficiency (Drost and Jonkers, 2009; McCarthy et al., 2007). Two recent studies conditionally deleted *Brca1* from hematopoietic cells (Santos et al., 2014; Vasanthakumar et al., 2016). One showed that leukemia cells transformed by MLL-AF9 exhibited reduced proliferation and increased differentiation in the absence of Brca1 (Santos et al., 2014). The second study showed that conditional *Brca1* deletion reduced blood cell counts and colony-forming progenitors. Transplantation of *Brca1*-deficient bone marrow cells into irradiated mice was associated with lower blood cell counts in recipient mice and a trend toward lower levels of donor cell reconstitution 10–15 days after transplantation. However, this study did not detect a significant reduction in HSC frequency and the consequences for the long-term reconstituting capacity of bone marrow cells was not assessed (Vasanthakumar et al., 2016). Therefore, it has not yet been tested whether Brca1 is required for HSC function or whether heterozygosity for *BRCA1* mutations affects recovery after chemotherapy in humans or in mice.

We evaluated the hematologic effects of chemotherapy on cancer patients with germline *BRCA1* or *BRCA2* mutations and found that in our small cohort, *BRCA1* mutations were associated with an increased risk of hematopoietic toxicity. Based on these clinical observations, we tested the effects of *BRCA1* mutations on hematopoiesis in mice. To do this, we characterized the effects of two different mutant *Brca1* alleles on mouse hematopoiesis. We show that *Brca1* is necessary for HSC maintenance and normal hematopoiesis but that different alleles exhibit differences in the severity of the HSC phenotype that do not correlate with differences in the severity of their effects on embryonic development.

RESULTS

Association of *BRCA1* mutations with hematopoietic toxicity from chemotherapy

Patients with germline *BRCA1* mutations commonly develop cancers that are treated with DNA-damaging chemotherapies. To test whether those patients are at increased risk for hematopoietic complications, we analyzed hematopoietic parameters in patients heterozygous for deleterious mutations in *BRCA1* at baseline (healthy patients) and after chemotherapy. We compared our patients to those who carry deleterious mutations in *BRCA2* for two reasons. First, it allowed for a comparison cohort that was similar in gender and age (Table 1), and second because prior data suggested that *BRCA2* mutation carriers may experience fewer episodes of neutropenia compared *BRCA1* mutation carriers (Shanley et al., 2006). In the latter report it was not possible to match for type of chemotherapy regimen and most of the patients did not receive doxorubicin, a standard component of

current breast cancer treatment. In our cohorts, most of the patients with *BRCA1* or *BRCA2* mutations that received chemotherapy had breast cancer and were treated with four cycles of dose dense doxorubicin plus cyclophosphamide (both drugs are DNA damaging) followed by four cycles of paclitaxel.

BRCA1 and *BRCA2* mutation carriers at our institution had normal blood cell counts at steady state (Figure 1a–f). Both sets of patients experienced hematopoietic toxicity after chemotherapy, though *BRCA1* mutation carriers tended to experience more frequent and severe hematopoietic toxicity from chemotherapy than *BRCA2* mutation carriers (Figure 1g–k). This was evident only in a subset of the patients as shown by the wide variations in the post-chemotherapy blood counts (Figure 1h–k; blood counts from each patient at the time of maximum neutrophil toxicity). Severity of maximal toxicity for blood parameters was also quantified as recommended by the NCI's criteria for adverse events (Figure 1l–o; Table 2). Since prophylactic G-CSF is standard for patients receiving dose dense doxorubicin plus cyclophosphamide and paclitaxel therapy, the overall rate of febrile neutropenia (neutropenia with fever) in breast cancer patients at our institution during the last three years has averaged ~5%. This is consistent with a recently reported 3.4% overall febrile neutropenia rate for this regimen in the United States (Caggiano et al., 2005). In contrast, 34% of *BRCA1* mutation carriers experienced febrile neutropenia, a significantly higher frequency than observed among *BRCA2* mutation carriers (0%; $P<0.0001$; Figure 1g). The *BRCA1* and *BRCA2* mutation carriers were relatively young patients (median age 41.5 and 46, respectively; Table 1) without co-morbidities, in whom febrile neutropenia would not be expected (Kouroukis et al., 2008). Consistent with the increased incidence of febrile neutropenia, we observed a trend toward an increased incidence of neutropenia among *BRCA1* mutation carriers as compared to *BRCA2* mutation carriers (Figure 1i; $P<0.1$).

BRCA1 mutation carriers also had a significantly higher frequency of grade 3/4 leukopenia (28% for *BRCA1* versus 8% for *BRCA2*; Figure 1l; $P<0.05$) and grade 3/4 lymphopenia (31% for *BRCA1* versus 0% for *BRCA2*; Figure 1n; $P<0.05$) as compared to *BRCA2* mutation carriers. There was also a trend toward an increased incidence of grade 3/4 anemia (31% for *BRCA1* versus 0% for *BRCA2*; Figure 1o) after chemotherapy. There were no differences in platelet counts between the two cohorts (**data not shown**).

There were no correlations of specific *BRCA1* mutations with febrile neutropenia. The small numbers of individuals with each mutation precluded our ability to determine whether or not specific mutations in the *BRCA1* gene were associated with toxicity (Table S1). Nevertheless, these data suggest that collectively germline *BRCA1* mutations are associated with a higher than expected risk for chemotherapy-associated hematopoietic complications.

Brca1 deficiency in mice causes pancytopenia

The high rate of hematopoietic toxicity in *BRCA1* mutation carriers after chemotherapy raised the question of whether reduced *BRCA1* function impairs the capacity to regenerate hematopoiesis after myeloablation. To test this, we generated *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mutant mice (Figure S1) (McCarthy et al., 2007). When the conditional *Brca1*$^{F22\text{-}24}$ allele is recombined, it is considered a null allele as mice that have homozygous deletion of this

allele in the germline die before birth, and conditional deletion in the breast epithelium leads to breast cancer (McCarthy et al., 2007). *Vav1*-iCre deletes in embryonic and adult hematopoietic cells, including HSCs (Georgiades et al., 2002). *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice at weaning had normal body weight and appeared healthy. As reported previously (McCarthy et al., 2007), there was no detectable Brca1 protein in spleen cells isolated from *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice (Figure 2a; top panel). Phosphorylation of H2AX was increased in *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ splenocytes, as would be expected for a functionally null DNA repair gene (Figure 2a; bottom panel).

Vav1-iCre;Brca1$^{F22\text{-}24/F22\text{-}24}$ mice developed severe pancytopenia. Three to six week old *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice had significantly decreased absolute numbers of white blood cells, including neutrophils and lymphocytes, as well as significantly reduced numbers of platelets compared to controls (Figure 2b–g). Deletion of a single allele of *Brca1* was sufficient to slightly but significantly reduce WBC and lymphocyte levels (Figure 2b and 2d).

Vav1-iCre;Brca1$^{F22\text{-}24/F22\text{-}24}$ mice had a shortened life-span and most died spontaneously without appearing ill, likely as a result of hematopoietic failure and its consequences (acute infection, bleeding etc.). Half of the *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice died by 75 days (Figure 2h). A fraction (27%; 7/26) of the *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice that survived beyond 3 months of age did become moribund (hunched, immobile, cold) prior to death and developed lymphocyte infiltrated splenomegaly (Figure S2a). Unlike the prior report that found p53 mutations in some spleens that were *Brca1* null (Vasanthakumar et al., 2016), when we used RNA-seq to analyze for mutations in expressed genes, only wild type p53 was found in these enlarged spleens. However, consistent with the same report, T-cell infiltration was present based on a decreased B-cell specific gene expression and an increased T-cell gene expression pattern in the enlarged spleens (Figure S2b). These data suggest that deletion of *Brca1* from mouse hematopoietic cells has the potential to promote the development of hematopoietic malignancies from surviving progenitors (Vasanthakumar et al., 2016).

Brca1 deficiency causes HSC depletion

To identify the cause of the pancytopenia in *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice, we examined hematopoiesis in their bone marrow. Six-week-old *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice had a significant reduction in bone marrow cellularity compared to littermate controls (Figure 3a; $P<0.05$). Strikingly, the bone marrow of *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice had only 3% of the $CD150^{+}CD48^{-}Lineage^{-}Sca\text{-}1^{+}ckit^{+}$ ($CD150^{+}CD48^{-}LSK$) (Kiel et al., 2005) HSCs observed in controls (Figure 3b and c). This decline in HSC frequency did not reflect HSC mobilization, as HSC frequency was not increased in the spleens of *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice (Figure 3d). Although there was a trend toward reduced HSC frequency in the heterozygotes (*Vav1-iCre;Brca1*$^{F22\text{-}24/+}$), the difference was not statistically significant. There was also a severe reduction in the frequency and absolute number of hematopoietic progenitor cells in the bone marrow, including $CD150^{-}CD48^{-}LSK$ multipotent progenitors (MPPs) (Oguro et al., 2013), $CD34^{+}CD16/32^{low}CD127^{-}Sca\text{-}1^{-}LK$ common myeloid progenitors (CMPs), $CD34^{+}CD16/32^{high}CD127^{-}Sca\text{-}1^{-}LK$ granulocyte

macrophage progenitors (GMPs), $CD34^{-}CD16/32^{low}CD127^{-}Sca\text{-}1^{-}LK$ megakaryocyte erythroid progenitors (MEPs) (Akashi et al., 2000) (Figure 3e-h, **S3b-e**), $B220^{+}IgM^{-}$ B cell progenitors, $B220^{+}IgM^{+}$ B cells (Figure 3i, **S3f**), and $CD3^{+}$ T cells (Figure 3j, **S3g**) in *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ bone marrow compared to controls. The frequency and absolute number of $CD11b^{+}Gr1^{+}$ myeloid cells (GM) was not significantly reduced in *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ bone marrow (Figure 3k, **S3h**). The frequency of $CD71^{+}Ter119^{+}$ erythroid progenitors was significantly increased in *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ bone marrow (Figure 3l), but absolute number were unchanged (Figure S3i). These data indicate that *Brca1* deficiency in *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice depletes HSCs and hematopoietic progenitor cells in young adult mice.

Competitive bone marrow transplantation assays were performed to functionally analyze HSCs from *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice (n=3 donors into 5 recipients each). Bone marrow cells from six week old *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ ($CD45.2^{+}$) mice and *Brca1*$^{F22\text{-}24/F22\text{-}24}$ ($CD45.2^{+}$) controls were each transplanted with equal numbers of wild-type congenic bone marrow cells ($CD45.1^{+}$) into lethally irradiated mice ($CD45.1^{+}$). Four weeks after transplantation, few donor derived *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ ($CD45.2^{+}$) cells were detected in the peripheral blood of recipient mice (Figure S3j). By 8 weeks after transplantation, there were no donor-derived B-, T- or myeloid cells in the peripheral blood of these recipients, some of which were followed out to 20 weeks without any detectable donor cell reconstitution (Figure S3k–m). Consistent with the decline in the frequency of HSCs based on surface marker phenotype (Figure 3b), *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice had no functional HSCs capable of reconstituting irradiated mice.

Heterozygosity for *Brca1* reduces HSC reconstituting capacity

To begin to determine if the bone marrow of *Vav1-iCre;Brca1*$^{F22\text{-}24/+}$ mice has increased sensitivity to DNA stress, we treated a cohort of *Vav1-iCre;Brca1*$^{F22\text{-}24/+}$ mice with two cycles of cyclophosphamide and monitored their blood counts for recovery abnormalities. Under these specific conditions, we did not observe consistent differences between heterozygous and wild type mice (Figure S3r–w). To observe more subtle differences, it may be necessary to either give different doses of cyclophosphamide, treat with several more drug cycles or treat with other drugs used in our patients such as cisplatin, doxorubicin and paclitaxel.

We have also tested heterozygous bone marrow sensitivity to proliferative DNA stress by evaluating whether haploinsufficiency for *Brca1* influences the bone marrow reconstituting capacity of HSCs. To do this bone marrow cells from six week old *Vav1-iCre;Brca1*$^{F22\text{-}24/+}$ ($CD45.2^{+}$) mice and *Brca1*$^{+/+}$ ($CD45.2^{+}$) controls were each transplanted with equal numbers of wild-type congenic bone marrow cells ($CD45.1^{+}$) into lethally irradiated mice ($CD45.1^{+}$). We observed no significant differences between *Vav1-iCre;Brca1*$^{F22\text{-}24/+}$ and control cells in the reconstitution of primary recipient mice (Fig. S3j–m). These results from challenges with chemotherapy and transplantation suggest that haploinsufficiency of *Brca1* in mice does not exhibit exceptionally high bone marrow sensitivity.

To further test if heterozygosity impaired HSC self-renewal potential, we serially transplanted bone marrow cells from the primary recipient mice into secondary recipient

mice. Sixteen weeks after primary transplantation, we transplanted bone marrow cells from primary recipient mice with levels of donor cell reconstitution (CD45.2+) nearest the median values in each treatment. *Vav1-iCre;Brca1*$^{F22\text{-}24/+}$ cells gave significantly lower levels of donor cell reconstitution in all lineages as compared to *Brca1*$^{+/+}$ control cells in secondary recipients (Figure S3n–q). These results suggest that *Vav1-iCre;Brca1*$^{F22\text{-}24/+}$ HSCs do in fact exhibit a reduced self-renewal capacity as compared to wild-type HSCs. Although single *Vav1-iCre* transgenic mice were not used as controls in this cohort, CBC abnormalities or defects in primary or secondary reconstitution by bone marrow cells from *Vav1-iCre* mice in the same pure C57/BL6 background have not been observed previously (Foley et al., 2013). These observations in mice are consistent with greater chemotoxicity in humans with *BRCA1* mutations and suggest that heterozygosity for a loss-of-function mutation in *Brca1* can impair the ability to regenerate hematopoiesis after one or more cycles of chemotherapy (Figure 1g).

Because serial bone marrow transplantation was required to observe a deleterious effect of proliferative stress on *Brca1* haploinsufficient HSCs, it remains possible that the heterozygous genotype does not impose as much chemotherapeutic toxicity as our human data would suggest. Since our patient data are from a retrospective analysis of a small cohort (Figure 1g), further work to prospectively observe more humans with inherited cancer predisposition mutations who are treated with chemotherapy is necessary. Also, generation and serial treatment of more *Brca1* haploinsufficient mice with various bone marrow stresses that include repeated cycles of cyclophosphamide, doxorubicin, cisplatin and paclitaxel will need to be completed.

Generation of wild-type and mutant knock-in alleles of human *BRCA1*

We and others have found that frameshift or stop-gain mutations in the last few exons of the *BRCA1* gene encode non-functional mutant proteins (Scully et al., 1999) expressed from messages that do not experience RNA decay (Perrin-Vidoz et al., 2002; Soyombo et al., 2013). These C-terminal mutations frequently lead to cancer phenotypes distinct from those caused by mutations elsewhere in the gene (Rebbeck et al., 2015). We have found that the common Ashkenazi Jewish *BRCA1* 5382insC founder mutation results in expression of a mutant transcript in the same amounts as *BRCA1* wild-type message in fibroblasts, induced pluripotent stem cells and teratomas (Soyombo et al., 2013). The 5382insC mutation leads to a C-terminal frame-shift mutation. We wondered whether this hypomorphic allele would have a less severe hematopoietic phenotype as compared to the null mutation.

To assess the 5382insC mutation's effects on embryogenesis and hematopoiesis, and to ensure that any abnormalities observed with humanization of the *Brca1* locus with the *BRCA1* 5382insC allele were due to the abnormal *BRCA1*, we generated mice that were humanized with wild-type human *BRCA1* in place of mouse *Brca1.* To do this, we designed a targeting vector that allowed for expression of both a wild-type *BRCA1* allele and, upon Cre mediated recombination, the *BRCA1* 5382insC mutation (Figure 4a; Figure S4a). C57BL/6 embryonic stem cells (ESCs) were electroporated with the targeting construct and screened for correctly targeted knock-in alleles (Figure S4b). Two lines that were correctly targeted and expressed human BRCA1 (Figure S4c, lanes 3–6) were also electroporated with

CMV-Cre to generate ESC lines that expressed the recombined *Brca1*^{5382insC} mutant allele (Figure S4c, lanes 7, 8). The *Brca1*^{BRCA1} allele substituted for wild-type mouse Brca1 function as evidenced by the fact that fully humanized homozygotes (*Brca1*$^{BRCA1/BRCA1}$) were born at Mendelian frequencies (Figure 4b, left) and remained alive and well with no hematopoietic abnormalities (Figure 4d–p) for up to 1.5 years of age (Figure S4d).

To generate mice with the *BRCA1* 5382inC allele (*Brca1*^{5382insC}) in the germline, *Brca1*$^{BRCA1/BRCA1}$ mice were mated with CMV-Cre deletor mice (Dupe et al., 1997). The progeny with recombination in the germline were then used for further analysis of mice who carried this human mutation. In contrast to the *Brca1*^{BRCA1} allele, homozygosity for the *Brca1*^{5382insC} allele was embryonic lethal, as 208 progeny from the heterozygous *Brca1*$^{5382insC/+}$ parents included no *Brca1*$^{5382insC/5382insC}$ homozygotes and an expected frequency of heterozygotes and wild type mice (Figure 4b, right, $p<0.0001$). This lethality confirms that the *5382insC* allele encodes a severe loss-of-function mutation. These data thus indicate that the wild-type human BRCA1 cDNA rescues mouse embryonic lethality (suggesting that alternative splicing is not necessary for this gene to function properly in mice), and that the *BRCA1* 5382insC mutant does not.

Hematopoiesis in mice with the *Brca1*^{5382insC} mutation

To assess the effects of the germline *Brca1*^{5382insC} mutant allele on hematopoiesis, we crossed the *Brca1*$^{5382insC/+}$ mice with the *Vav1-iCre;Brca1*$^{F22-24/+}$ mice to generate *Vav1-iCre; Brca1*$^{F22-24/5382insC}$ biallelic mutant mice. This is a similar genetic configuration predicted to occur in many human cancers—a germline mutation (*Brca1*^{5382insC}) in one allele followed by somatic loss of heterozygosity as a result of deletion of the second allele. The biallelic *Vav1-iCre; Brca1*$^{F22-24/5382insC}$ mice were healthy at weaning. In contrast to the severe hematopoietic defects in *Vav1-iCre;Brca1*$^{F22-24/F22-24}$ mice, average peripheral blood counts (Figure S5a–f) and bone marrow stem and progenitor cell frequencies were normal in adult *Vav1-iCre; Brca1*$^{F22-24/5382insC}$ mice (Figure S5h-p). However, the normal blood counts may be attributed to the main presence of cells that lack recombination of the floxed null allele (Figure S5g). In contrast to the presence of only non-recombined hematopoietic cells in *Vav1-iCre; Brca1*$^{F22-24/5382insC}$ mice (same amplification curve as obtained from DNA derived from Cre-negative *Brca1*$^{F22-24/+}$ control bone marrow), hematopoietic cells from *Vav1-iCre;Brca1*$^{F22-24/F22-24}$ mice exhibited significant recombination (Figure S5g). A possible explanation for the lack of somatic recombination of the *Brca1*$^{F22-24}$ allele when the germline allele is *Brca1*^{5382insC}, is that the BRCA1^{5382insC} protein is more deleterious to hematopoietic cells than the simple null allele, and thus the only cells that survived into adulthood were those that were not somatically recombined.

To test this, we used *Mx1-Cre* to conditionally recombine the floxed allele in the adult hematopoietic system. The un-induced *Mx1-Cre;Brca1*$^{F22-24/F22-24}$ biallelic mice were healthy at weaning but when *Mx1-Cre* was induced with pIpC at 4 weeks of age, these mice experienced fully penetrant, rapid morbidity and mortality In contrast, *Mx1-Cre;Brca1*$^{F22-24/F22-24}$ mice did not (Figure 5a). To confirm there were no differences in recombination we generated mice with the null mutation in the germline. To do this *Brca1*$^{F22-24/F22-24}$ mice were mated with CMV deletor mice (Dupe et al., 1997). The

progeny with recombination in the germline (*Brca1*$^{\Delta/+}$) were then used to generate Mx1-Cre;*Brca1*$^{F22\text{-}24/\Delta}$ biallelic mice to compare to Mx1-Cre;*Brca1*$^{F22\text{-}24/5382insC}$ biallelic mutant mice. Again, the Mx1-Cre;*Brca1*$^{F22\text{-}24/\Delta}$ biallelic mice survived while the Mx1-Cre;*Brca1*$^{F22\text{-}24/5382insC}$ biallelic mutant mice did not (Figure 5a). These data indicate that the *Brca1*$^{\Delta/5382insC}$ genotype is more deleterious to hematopoietic cells than the *Brca1*$^{\Delta/\Delta}$ genotype.

The rapid mortality in pIpC-treated *Mx1-Cre; Brca1*$^{F22\text{-}24/5382insC}$ mice was associated with severe pancytopenia. The organs of the pIpC induced *Mx1-Cre;Brca1*$^{F22\text{-}24/5382insC}$ mice were pale (**data not shown**). As expected, for both the *Mx1-Cre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ and *Mx1-Cre;Brca1*$^{F22\text{-}24/5382insC}$ mutant mice in Figure 5a, there were significantly decreased absolute numbers of white blood cells, including neutrophils and lymphocytes, as well as significantly reduced numbers of platelets (Figure 5b–f). However, *Mx1-Cre; Brca1*$^{F22\text{-}24/5382insC}$ mice had a significantly more severe pancytopenia than *Mx1-Cre;Brca1*$^{F22\text{-}24/\Delta}$ mice with the lowest absolute numbers of white blood cells, including neutrophils, monocytes and lymphocytes, as well as reduced numbers of platelets and hemoglobin (Figure 5b–g).

The severe anemia in the *Mx1-Cre; Brca1*$^{F22\text{-}24/5382insC}$ mice was the largest and most significant difference from the *Mx1-Cre;Brca1*$^{F22\text{-}24/\Delta}$ mice (Figure 5g). Since the half-life of mouse red blood cells in wildtype mice has been measured at more than 20 days (Van Putten, 1958), it is possible there was bleeding secondary to the severe thrombocytopenia. In fact, in two mice that were necropsied immediately after death, we observed large pools of blood in the abdominal and thoracic cavities. Thrombocytopenia can occur quickly with loss of progenitors as mouse platelet half-life has been estimated to be 3.4 days (Jayachandran et al., 2010).

To further characterize pIpC-induced *Mx1-Cre;Brca1*$^{F22\text{-}24/5382insC}$ mice, we examined hematopoiesis in their bone marrow. Six-week old *Mx1-Cre;Brca1*$^{F22\text{-}24/5382insC}$ mice that had been treated with pIpC at 4 weeks of age had a severe reduction in HSCs and early progenitors compared to pIpC-treated controls (Figure 5h–l; $P<0.01$). In fact, the bone marrow of pIpC-treated *Mx1-Cre;Brca1*$^{F22\text{-}24/5382insC}$ mice had no detectable HSCs (Figure 5h). Hematopoietic progenitors including MPPs, CMPs, GMPs and MEPs were also not detectable in the bone marrow of *Mx1-Cre;Brca1*$^{F22\text{-}24/5382insC}$ (Figure 5i–l). When compared with hematopoiesis in *Mx1-Cre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice (Vasanthakumar et al., 2016) (data not shown), a more severe hematopoietic defect in *Mx1-Cre;Brca1*$^{F22\text{-}24/5382insC}$ mice is present, suggesting that the BRCA1^{5382insC} protein is more deleterious to hematopoietic stem and progenitor cells than the null allele.

DISCUSSION

In this manuscript, we show a cohort of patients with *BRCA1* mutations experienced increased hematopoietic toxicity and complications after cancer chemotherapy. We also observed that *Brca1* is required for HSC function and normal hematopoiesis in mice. When *Brca1* was conditionally deleted from embryonic hematopoietic cells, young adult mice developed pancytopenia (Figure 2) and a loss of nearly all HSCs (Figure 3). Moreover,

heterozygosity for a loss-of-function allele of *Brca1* in mouse hematopoietic cells led to a slight but significant decrease in white blood cells and lymphocytes (Figure 2) as well as deficits in HSC reconstituting potential upon serial transplantation (Figure S3).

These results are consistent with a reduced hematopoietic regenerative capacity in *BRCA1* heterozygous humans after chemotherapy, suggesting that even a partial loss of BRCA1 function reduces the capacity for hematopoietic recovery due to direct DNA damage or replication stress after myeloablation. The concept that replication stress leads to more chemotherapeutic toxicity for *BRCA1* mutation carriers is also consistent with prior work that has suggested there is enhanced replication stress (due to decreased stalled fork repair) in *BRCA1* heterozygous epithelial cells. This abnormality in heterozygous cells was hypothesized to enhance the formation of tumors in epithelial cells (Pathania et al., 2014).

These data suggest a “cell death or transformation” tissue specificity hypothesis and could explain why patients with germline *BRCA1* mutations have a predisposition to epithelial cancers, but do not have a predisposition to hematological malignancies. The ultimate loss of *BRCA1* heterozygosity, which is promoted by diminished DNA repair in the heterozygous state and is thought to be required for transformation of epithelial cells to cancer (Pathania et al., 2014), is not tolerated by hematopoietic stem cells.

This is the first report of generation and characterization a humanized *Brca1* allele. The human *BRCA1* cDNA was knocked into the mouse *Brca1* locus to study its function. Humanization of mouse genes has proven useful for *in vivo* functional evaluation of human p53 mutations (Song et al., 2007). Like p53, the introduction of human mutations into the mouse *Brca1* allele is advantageous as there are significant differences in amino acid sequence between mouse Brca1 and human BRCA1. The mouse protein is only 60% identical to the human BRCA1 protein (Sharan et al., 1995). Our finding of embryonic lethality for the $Brca1^{5382insC/5382insC}$ genotype but not in un-recombined $Brca1^{BRCA1/BRCA1}$ mice (Figure 4), confirms that human BRCA1 can perform many of the necessary functions of mouse Brca1 after being knocked into the mouse *Brca1* locus.

Conditional deficiency for *Brca1* using Mx1-Cre in adult mice has previously been reported to increase differentiation in MLL-AF9 induced leukemia (Santos et al., 2014), diminish hematopoietic cell proliferation *in vitro*, and lead to mild leukopenia and anemia (Vasanthakumar et al., 2016). However, neither of these studies reported a deleterious effect of *Brca1* deficiency on HSC frequency or function. Use of the Vav1-iCre allele to delete *Brca1* in embryonic and adult HSCs and use of the new human $Brca1^{5382insC}$ allele were not part of the prior studies. The use of different Cre alleles suggests that deletion of *Brca1* in the embryonic HSCs (Vav1-iCre) may be more deleterious than deletion in adult HSCs (Mx1-Cre).

A trivial explanation for why an HSC defect was only observed in mice using the Vav1-iCre allele for conditional deletion of *Brca1* is that there was more recombination in HSCs with the Vav1-iCre allele compared to those with the Mx1-Cre allele. Although this explanation is not possible to confirm or refute without analysis of the original mice, because

Vasanthakumar et al did provide evidence for full recombination in hematopoietic cells from their Mx1-Cre transgenic mice, it is an unlikely explanation (Vasanthakumar et al., 2016).

Different *BRCA1* mutations have been shown to have distinct effects on cancer phenotypes. Humans with mutations at the extreme C- and N-terminus of *BRCA1* experience more breast cancer and less ovarian cancer as compared to humans with mutations in the middle of the *BRCA1* gene (Rebbeck et al., 2015). Our surprising observation that the $Brca1^{5382insC}$ mutation led to a more severe adult hematopoietic phenotype than the *Brca1* null mutation (Figure 5) suggests that distinct germline *BRCA1* mutations may result in different degrees of chemotherapeutic toxicity, as well. More patients with these mutations are needed to make associations and more investigation into the mechanism of this increased toxicity of the $Brca1^{5382insC}$ allele in the mice will be important.

Because the $Brca1^{5382insC}$ allele, in contrast to the null allele, expresses a mutant protein (Figure S4c **vs.** Figure 2a), it could indeed have hypo- or hyper-morphic effects on cells. Expression of the *BRCA1* 5382insC mutant mRNA from humans heterozygous for the *BRCA1* 5382insC mutation is equivalent to the expression of the wild type mRNA from the other allele in primary human fibroblasts, induced pluripotent stem cells and teratomas. However, the expression of BRCA1 5382insC in these cells does not promote excessive cell death, differentiation, survival or growth (Soyombo et al., 2013). Further, heterologous expression of this mutant BRCA1 in cell lines does not lead to altered growth or survival (**data not shown**). These observations may be due to the expression from the normal *BRCA1* allele. The mutant protein may only be detrimental in a completely deficient BRCA1 background. Further studies to understand why the *BRCA1* 5382insC allele leads to an *in vivo* phenotype distinct from the *Brca1* null allele are necessary.

Stem cells are susceptible to DNA damage due to their longevity and self-renewal potential. HSCs from mice with mutations in DNA damage repair proteins that also lead to cancer susceptibility syndromes, such as *Brca2* (Navarro et al., 2006) and *Msh2* (Reese et al., 2003), have defects in their ability to reconstitute bone marrow in irradiated mice, and mice with mutant *Rad50* exhibit hematopoietic failure (Bender et al., 2002). However, the hematopoietic phenotype we observed after *Brca1* deletion is much more severe than the phenotypes reported in these studies.

Several mouse models have been generated to study *BRCA1*-mutant breast cancer (Dine and Deng, 2013; Drost et al., 2011; Drost and Jonkers, 2009; Evers and Jonkers, 2006; Shakya et al., 2011). These models confirm that Brca1 maintains genome stability *in vivo,* and that without normal Brca1 in breast epithelial tissues, breast tumorigenesis occurs. However, breast cancer develops in *Brca1* knockout mice only after a long latency (even if p53 is also deficient). This is consistent with the fact that human *BRCA1* mutation carriers are only diagnosed with cancer as adults, if ever.

Here we describe mice with different *Brca1* alleles mutated specifically in the hematopoietic system that have distinct phenotypes, which, in contrast to the breast cancer phenotype, occur rapidly and are fully penetrant (for allele/phenotype summary see Table S2). In addition to the new information about the role of Brca1 in hematopoiesis, these allele

combinations provide the field with powerful tools for rapid investigation of the pathogenicity of *BRCA1* variants of unknown significance.

Finally, given the potent requirement for *Brca1* in HSCs, an inherited *BRCA1* mutation may be a marker to add to the list of patient risk factors, such as age and co-morbidities (Caggiano et al., 2005), that support the prophylactic use of growth factors and antibiotics, and close monitoring for chemotherapy-related hematopoietic complications. Preventative use of myeloid growth factor support may, however, be counterproductive if unrepaired replication-induced mutations are increased in *BRCA1* heterozygotes. Prophylactic growth factor support and antibiotics should, therefore, be evaluated prospectively in *BRCA1* mutation carriers who are receiving chemotherapy.

METHODS

Patients

A list of patients with *BRCA1* or *BRCA2* mutations treated between January 1, 2011 and October 31, 2014, were identified from the University of Texas Southwestern Medical Center's Cancer Genetics database. Patients were categorized based on cancer type and chemotherapy treatments. A retrospective chart review was then conducted on these patients to collect information on patient characteristics (Table 1), as well as comorbidities and past medical/surgical histories, type of cancer, age of diagnosis, treatment, treatment complications (if applicable), and complete blood cell counts. For any patient who had at least one complete blood cell count recorded in their medical record, baseline complete blood cell count values were selected for each patient based on the following criteria: pre-treatment (but as close to beginning of therapy as possible within 5 years of cancer diagnosis), no active infection, no procedural context (e.g. post-biopsy or post-operative), and did not appear to be an outlier if other complete blood cell counts were available for comparison.

For the patients who underwent chemotherapy for their cancer, the most severe adverse hematopoietic event during chemotherapy and its associated toxicity score was recorded. Grades of blood cell count toxicity were assigned based on the National Cancer Institute Common Terminology Criteria for Adverse Events v3.0 guidelines (Trotti et al., 2003) (Table 2). Neutropenic fever was defined as an absolute neutrophil count < 500 cells/mm^3 and fever. Fever was defined as a single oral temperature of >38.3°C (101°F) or a temperature of >38.0°C (100.4°F) sustained for more than one hour.

Following collection of these data, statistical analyses were conducted on de-identified data. Range, mean, and standard deviations of complete blood cell components (neutrophils, platelets, hemoglobin, etc.) in *BRCA1/2* mutation carriers were compared to the normal ranges. Analysis of variations in complete blood cell components in response to different chemotherapy regimens were also evaluated for differences between the *BRCA1* and *BRCA2* mutant patients. This study (STU 072014-043; Analysis of Complete Blood Counts (CBCs) in BRCA Mutation Carriers) was approved by the University of Texas Southwestern Medical Center Institutional Review Board.

Mice

The *Brca1*$^{F22\text{-}24}$ (McCarthy et al., 2007), *Mx1-Cre* (Rajewsky et al., 1996) and *Vav1-iCre* (Georgiades et al., 2002) alleles, all on a C57/BL6 pure background, have been previously described and were obtained from Jackson Labs. C57BL/Ka-Thy-1.2 (CD45.1) mice were used as transplant recipients. Both male and female mice between 6–14 weeks old were used in all studies. Mx1-Cre was induced as describe previously (Oravecz-Wilson et al., 2009).

The *BRCA1* knock-in mice were generated as described in detail in the **Extended Experimental Procedures** and Figure S4. Briefly, the targeting vector was constructed to generate a knock-in allele that conditionally generated the *BRCA1* 5382insC mutation as well as constitutively humanized *BRCA1* mice (Figure 4A and Figure S4).

All *Brca1* mutant mice were genotyped from tail snips using real-time PCR assays designed by and available from Transnetyx. The assays were designed to detect the wild-type and mutant alleles in the presence or absence of recombination. Mice were housed in the Unit for Laboratory Animal Medicine at the University of Texas Southwestern Medical Center under specific pathogen-free conditions and were monitored regularly for evidence of disease and abnormal peripheral blood cell counts. The animal use protocol was approved by the University of Texas Southwestern Institutional Animal Care and Use Committee (APN 2011-0143).

Bone Marrow Transplantation

Adult recipient mice (CD45.1) were administered a minimum lethal dose of radiation using an XRAD 320 X-ray irradiator (Precision X-Ray Inc.) to deliver two doses of approximately 540 rad (1,080 rad in total) at least 3 hours apart. Cells were injected into the retro-orbital venous sinus of anesthetized recipients. For competitive bone marrow transplants 5 x 10^5 donor and 5 x 10^5 recipient cells were transplanted. Blood was obtained from the submandibular plexus of recipient mice at the indicated time points after transplantation. Red blood cells were lysed with ammonium chloride potassium buffer. The remaining cells were stained with antibodies (Tonbo Biosciences, Inc.) against CD45.2, CD45.1, CD45R (B220), CD11b, CD3, and Gr-1 to assess donor cell engraftment. Mice that died were omitted from the analyses.

Hematopoietic Analysis

Bone marrow cells were isolated by flushing the long bones (femurs and tibias) in Ca^{2+} and Mg^{2+} free Hank's buffered salt solution (Corning) supplemented with 3% heat-inactivated bovine serum (Gibco). Spleens were prepared by crushing tissues between frosted slides. Cell number and viability were assessed by a Vi-CELL cell viability analyzer (Beckman Coulter) or by counting on a hemocytometer.

Flow cytometric analysis of specific hematopoietic progenitors was performed as previously described (Foley et al., 2013; Signer et al., 2014). Complete blood cell count analysis was performed on peripheral blood using the Hemavet 950 with MULTI-TROL Mouse as an equilibration control (Drew Scientific).

Western blot analysis

Mouse tissues were lysed in RIPA buffer (Cell Signaling Technology). 100 μg of total protein was electrophoresed on 6% SDS-PAGE gels, and transferred to a PVDF membrane. Proteins were detected with anti-Brca1 antiserum (1:100; GH118, kind gift of Dr. Jos Jonkers), mouse monoclonal anti-Hip1 (1:1000; 1B11, Ross laboratory (Ames et al., 2013)) and rabbit polyclonal anti-γH2AX (1:1000, Cell Signaling). Blots were developed with Supersignal West Pico chemiluminescence substrate (Pierce).

Statistical Analysis

Statistical significance was assessed using a two-tailed Student's t-test with p values (*P<0.05, **P<0.01, ***P<0.001). A Fisher's Exact test (*P<0.05) was used to asses statistical significance in Figure 1 (G, L–O). For Kaplan Meier curves depicting survival analyses, a log-rank test was used. All statistical analyses were performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. All RNA sequencing expression data and accession codes can be found at the Gene Expression Omnibus: GSE91390.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Mouse hematopoietic stem cells (mHSCs) require normal Brca1 to survive.
- Knocked-in wild-type human *BRCA1* cDNA fully substitutes for mouse *Brca1*.
- A *BRCA1*/5382insC mutation is more deleterious to mHSCs than a *Brca1* null allele.

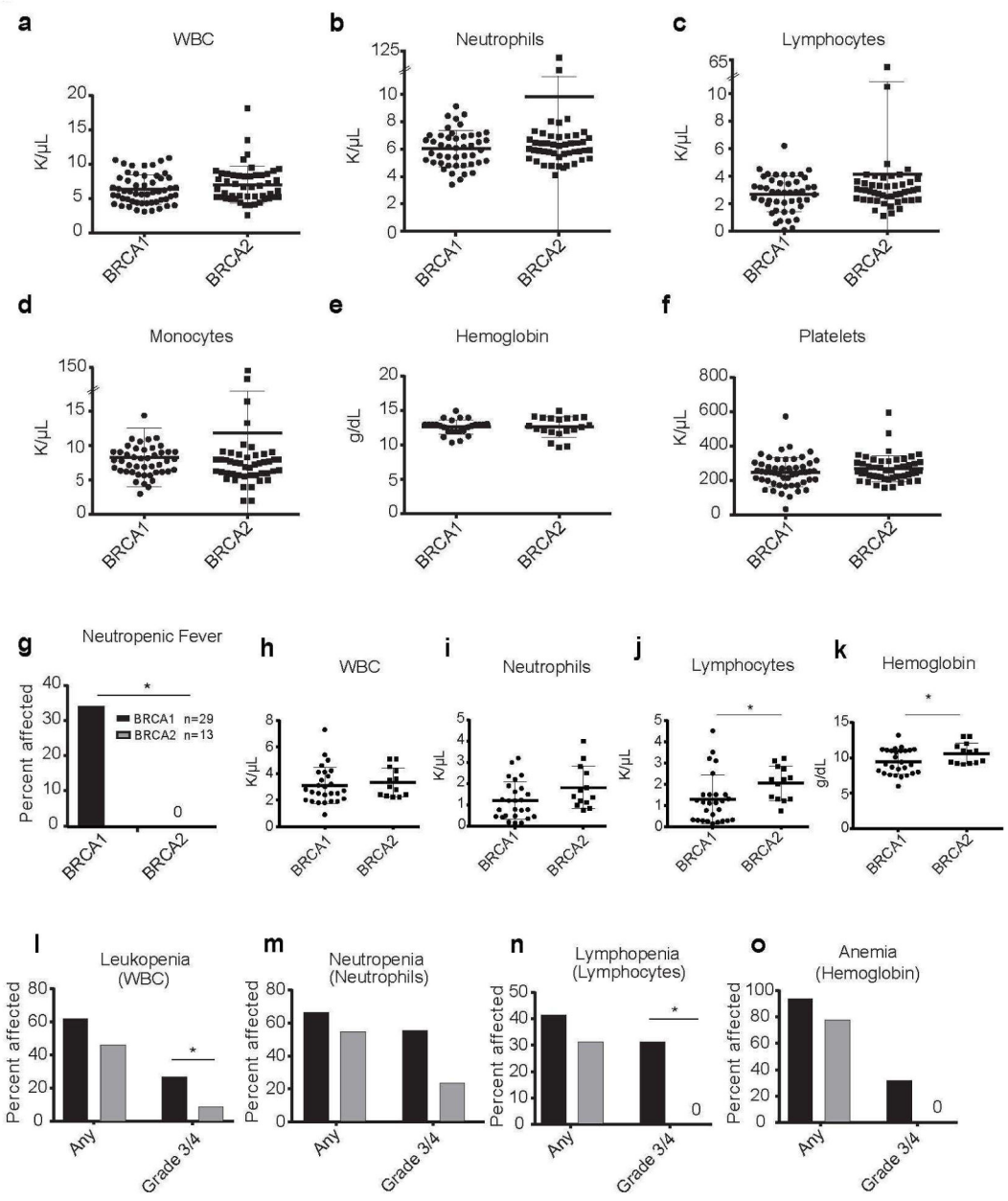

Figure 1. Cancer patients with *BRCA1* mutations have increased hematopoietic toxicity after chemotherapy

(**A–F**) Complete blood cell counts from individuals with *BRCA1/2* mutations who had not been exposed to chemotherapy. No significant differences were observed in (A) white blood cells (WBC), (B) Neutrophils, (C) Lymphocytes, (D) Monocytes, (E) Hemoglobin, or (F) Platelets between *BRCA1* and *BRCA2* mutation carriers (n= 63 *BRCA1* and 50 *BRCA2* patients). Bars in (A–F) represent means ± standard deviation. (G) Frequency of febrile neutropenia (FEN) in *BRCA1* or *BRCA2* cancer patients treated with chemotherapy. (**H–K**) Parameters from blood counts with the lowest absolute neutrophil counts after *BRCA1* or *BRCA2* patients were treated with chemotherapy. (**L–O**) Frequency and severity of hematopoietic toxicity in *BRCA1* or *BRCA2* cancer patients treated with chemotherapy.

Black bar = *BRCA1* mutation carriers. Grey bar = *BRCA2* mutation carriers. Percent of patients affected with any grade and severe grade 3 or grade 4 (L) leukopenia (M) neutropenia, (N) lymphopenia or (O) anemia are shown. Grades of blood cell count toxicity were assigned as standardized by the National Cancer Institute. Common Terminology Criteria for Adverse Events v3.0 (Trotti et al., 2003). Neutropenic fever was defined as an ANC < 500 cells/mm^3 and a sustained fever > 38°C (100.4°F). Statistical significance in (A–F, H–K) was assessed using a two-tailed Student's t-test and in (G, L–O) using a Fisher's Exact test (*$P<0.05$).

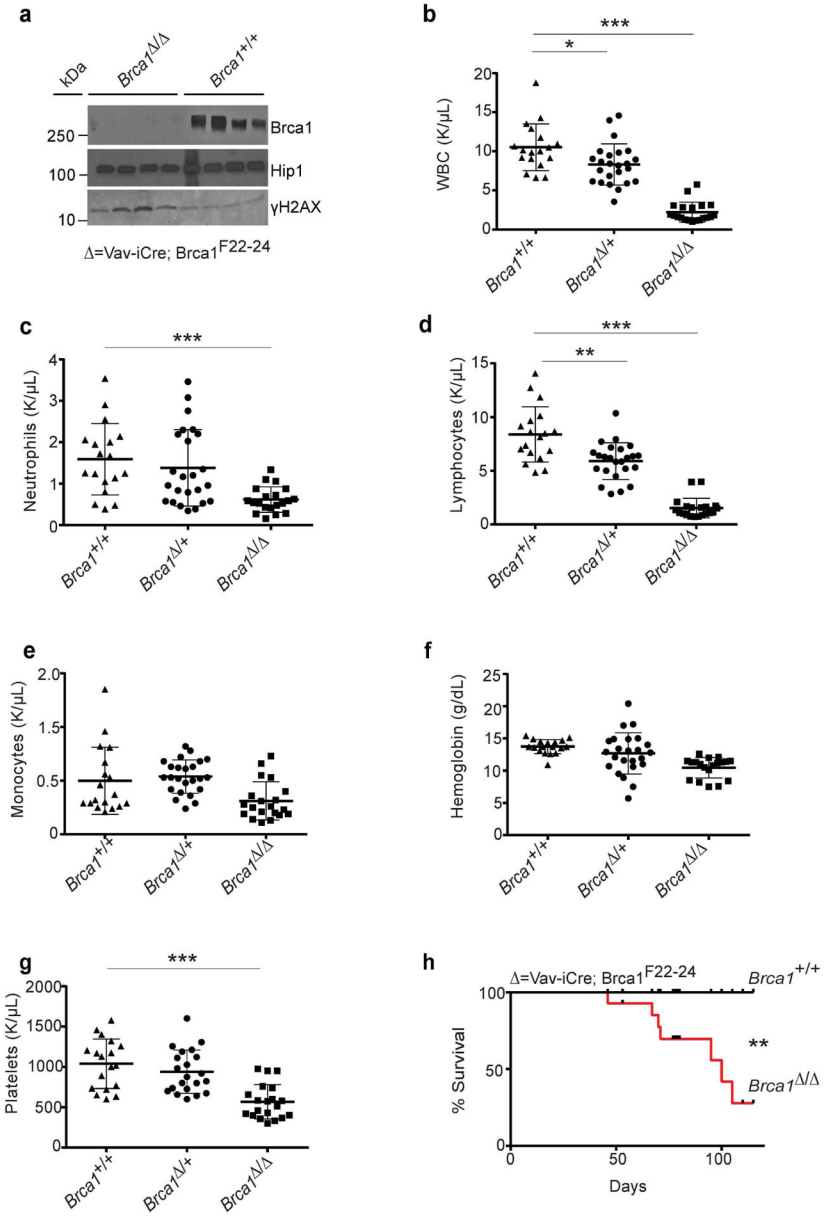

Figure 2. *Brca1* deficiency leads to severe hematologic abnormalities

(**A**) Western blot analysis for mouse Brca1, Hip1 (loading control) and γH2AX in spleen extracts (100 μg per lane) from *Vav1-iCre;Brca1*$^{F22-24/F22-24}$ (Δ/ Δ; n=4) and control (+/+; n=4) mice. (**B–G**) Blood counts from 3–6 week old wild-type control mice (+/+; n=18), *Vav1-iCre;Brca1*$^{F22-24/+}$ (Δ/+; n=24) and *Vav1-iCre;Brca1*$^{F22-24/F22-24}$ (Δ/ Δ; n=20) mice. (B) White blood cells (WBC), (C) neutrophils, (D) lymphocytes, (E) monocytes, (F) hemoglobin, and (G) platelets are shown. (**H**) Kaplan-Meier survival curve of control (black line; +/+; n=14) and *Vav1-iCre;Brca1*$^{F22-24/F22-24}$ (red line; Δ/ Δ; n=14) mice. All data represent means ± standard deviation. Statistical significance was assessed using a two-tailed Student's t-test except in (H) where a log-rank test was used (*P<0.05, **P<0.01, ***P<0.001).

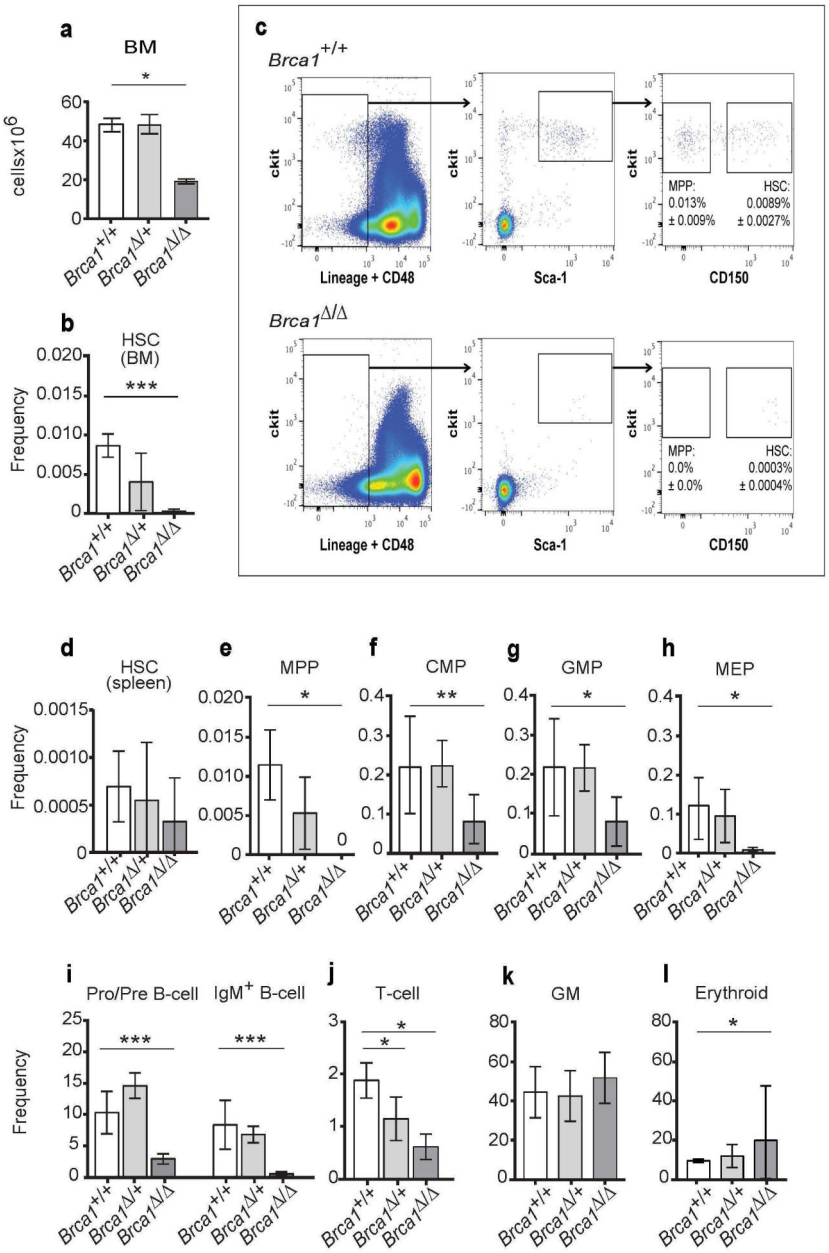

Figure 3. *Brca1* deficiency causes HSC depletion

(**A**) Bone marrow (BM) cellularity (1 femur and 1 tibia) of control (+/+; n=4), *Vav1-iCre;Brca1*$^{F22\text{-}24/+}$ (Δ/+; n=5), and *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice (Δ/Δ; n=4). (**B**) Frequency (number of HSCs/total BM cells) of HSCs in the BM of control (+/+; n=7; white bar), *Vav1-iCre;Brca1*$^{F22\text{-}24/+}$(Δ/+; n=5; light grey bar), and *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice (Δ/Δ; n=4; dark grey bar). (**C**) Representative flow cytometry plot showing CD150^{+}CD48^{-}LSK HSCs in wild type mice (+/+; top panel) and *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice (Δ/Δ; bottom panel). Frequency of (**D**) HSCs in the spleen of control (n=3), *Vav1-iCre;Brca1*$^{F22\text{-}24/+}$ (n=3), and *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice (n=3). Frequency of (**E**) MPPs, (**F**) CMPs, (**G**) GMPs, (**H**) MEPs, (**I**) B lineage cells, (**J**) T lineage

cells, (**K**) myeloid cells, and (**L**) erythroid cells in the bone marrow of control (+/+; n=7; white bar), *Vav1-iCre;Brca1*$^{F22\text{-}24/+}$(Δ/+; n=5; light grey bar), and *Vav1-iCre;Brca1*$^{F22\text{-}24/F22\text{-}24}$ mice (Δ/Δ; n=4; dark grey bar). Statistical significance was assessed using a two-tailed Student's t-test (*P<0.05, **P<0.01, ***P<0.001).

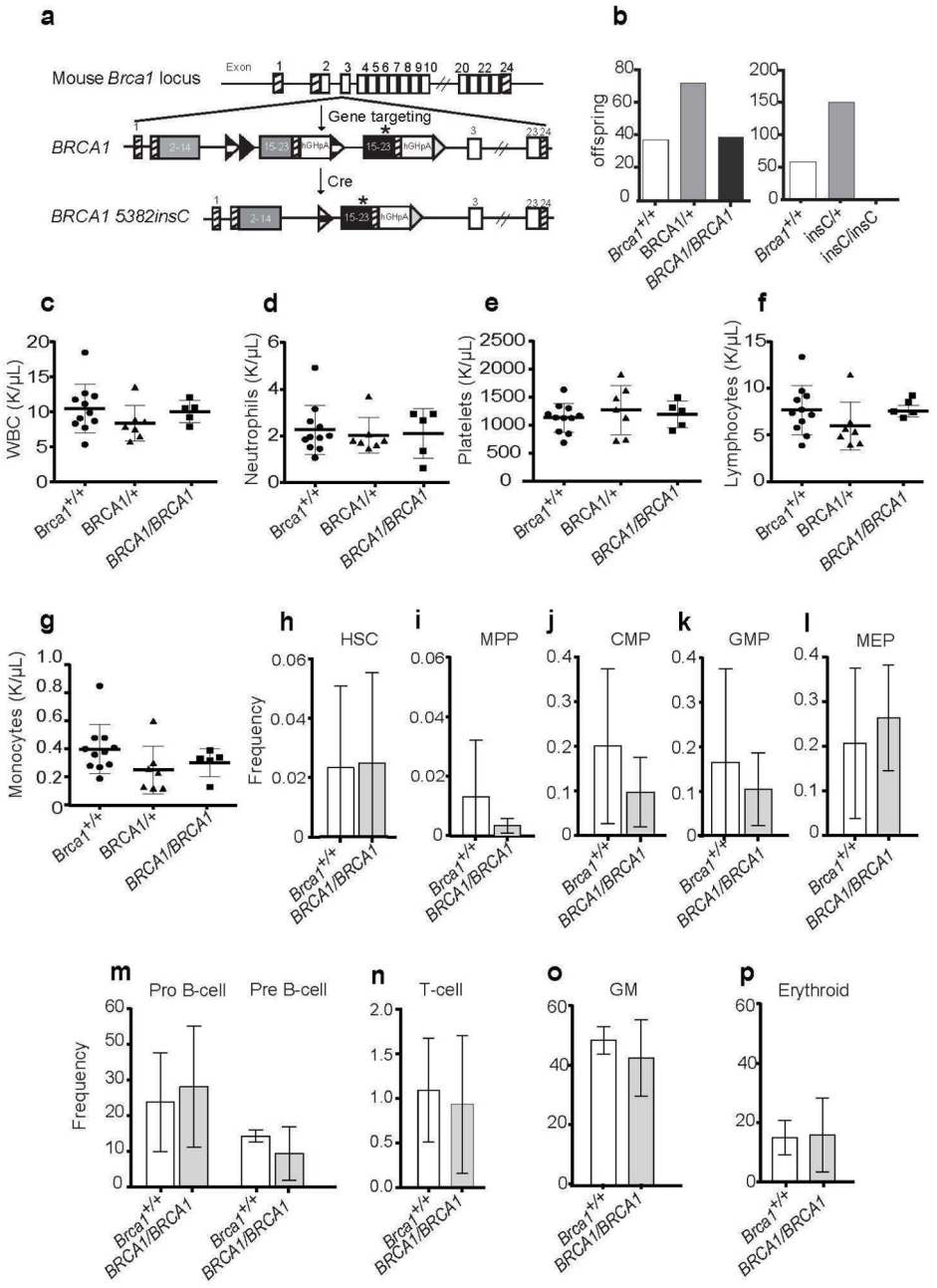

Figure 4. Humanization of the *Brca1* allele with wild type *BRCA1* or *BRCA1 5382insC* knocked-in cDNA sequences

(**A**) Targeting vector used to knock *BRCA1* into the *Brca1* locus. This allowed for humanization of the *Brca1* gene with a wild type *BRCA1* or the *BRCA1* 5382insC point mutation. **(B)** *BRCA1* 5382insC knock-in is embryonic lethal. *(Left)* Number of offspring and the genotypes produced from 15 heterozygous $Brca1^{BRCA1/+}$ mating pairs. *(Right)* Number of offspring and the genotypes produced from 30 heterozygous $Brca1^{5382insC/+}$ (InsC) mating pairs. **(C–G)** Blood counts from wild-type control mice (+/+; n=11), homozygous $Brca1^{huBRCA1/huBRCA1}$ (*BRCA1/BRCA1*; n=5) and heterozygous

Brca1$^{huBRCA1/+}$ (*BRCA1/+*; n=7). (C) White blood cells (WBC), (D) neutrophils, (E) platelets, (F) lymphocytes and (G) monocytes are shown.(**H**) HSCs, (**I**) MPPs, (**J**) CMPs, (**K**) GMPs (**L**) MEPs (**M**) B lineage cells, (**N**) T lineage cells, (**O**) myeloid cells, and (**P**) erythroid cells in the bone marrow of control (+/+; n=3; white bar) and *Brca1$^{huBRCA1/huBRCA1}$* (*BRCA1/BRCA1*; n=3; light grey bar). Statistical significance was assessed using a two-tailed Student's t-test. There were no significant differences between the genotypes.

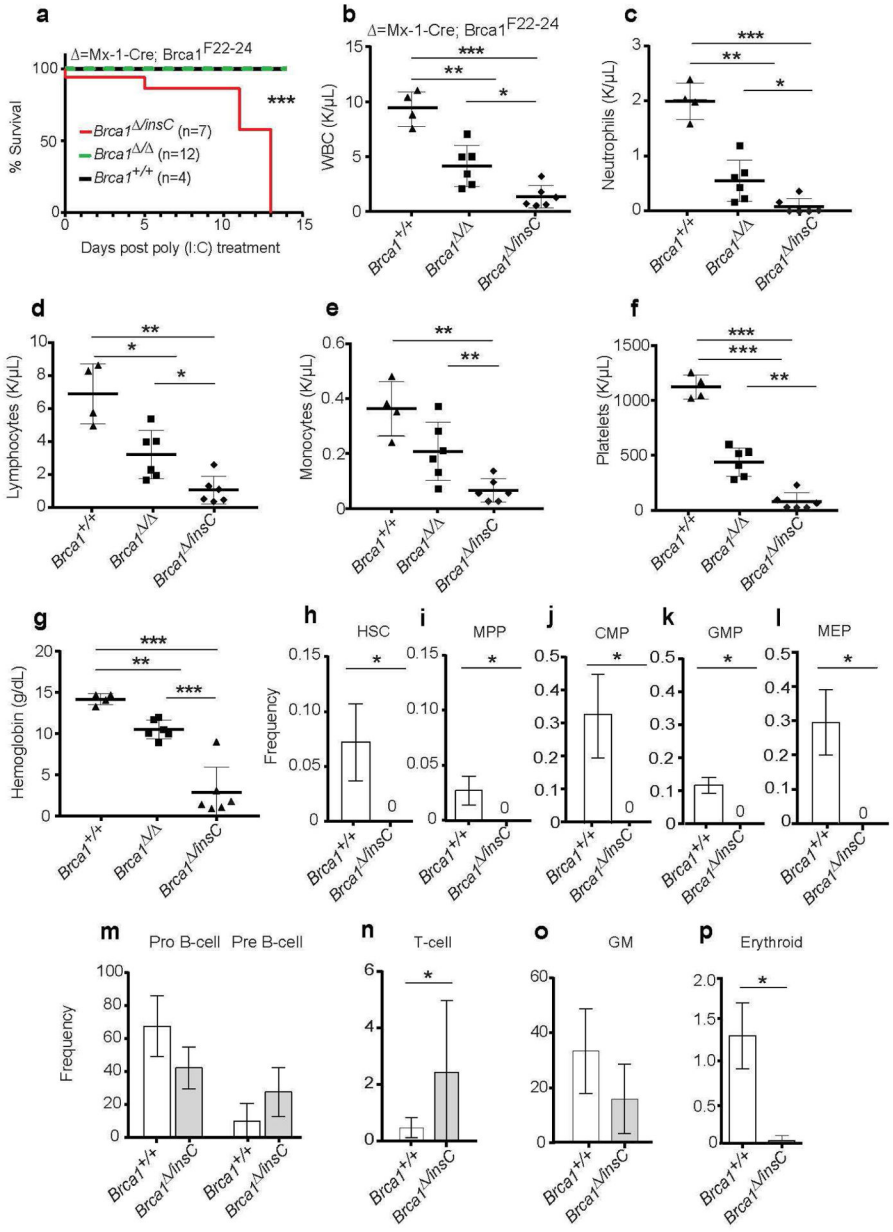

Figure 5. Substitution of mouse *Brca1* with the human *BRCA1* 5382insC generates a compound heterozygote with a more severe hematopoietic phenotype than homozygous *Brca1* null mice

(**A**) Kaplan-Meier survival curve of control (black line; *Brca1*+/+; n=4), *Mx1-Cre;Brca1*$^{F22-24/\ F22-24}$ (n=7) plus *Mx1-Cre;Brca1*$^{F22-24/\Delta}$ (n=5) (green dashed line; *Brca1*$^{\Delta/\ \Delta}$) and *Mx1-Cre;Brca1*$^{F22-24/5382insC}$ (red line; *Brca1*$^{\Delta/insC}$; n=7) mice. (**B–G**) Complete blood cell counts from wild-type mice (*Brca1*$^{+/+}$; n=4), *Mx1-Cre;Brca1*$^{F22-24/F22-24}$ (n=4 mice) plus *Mx1-Cre;Brca1*$^{F22-24/\Delta}$ (n=5) mice (*Brca1*$^{\Delta/\Delta}$) and *Mx1-Cre;Brca1*$^{F22-24/5382insC}$ (*Brca1*$^{\Delta/insC}$; n=7 mice) (**B**) White blood cells (WBC), (**C**) neutrophils, (**D**) lymphocytes, (**E**) monocytes, (**F**) platelets, and (**G**) hemoglobin are shown. All data represent means ± standard deviation. (**H**) HSCs, (**I**) MPPs, (**J**) CMPs, (**K**) GMPs, (**L**) MEPs (**M**) B lineage cells, (**N**) T lineage cells, (**O**) myeloid cells, and (**P**) erythroid cells

in the bone marrow of control (+/+; n=3; white bar) and *Mx1-Cre;Brca1*$^{F22\text{-}24/5382insC}$ (Δ/+; n=3; light shaded bar). Statistical significance was assessed using a two-tailed Student's t-test except in (**A**) where a log-rank test was used (*P<0.05, **P<0.01, ***P<0.001).

Table 1

Characteristics of human *BRCA1 and BRCA2* mutation carriers

Population parameters		***BRCA1***	***BRCA2***	**Total**
Patients (n)	Total	104	96	200
	Female	90	87	177
	Male	14	9	23
Age	Range	20–79	20–77	-
	Median	42	46	-
Race (n)	Black	23	17	40
	Asian	8	7	15
	Caucasian	33	44	77
	Hispanic	40	28	68
Cancer (n)	Total	45	23	68
	Breast *	33	17	50
	Ovarian	7	1	8
	Other	5	5	10
Chemo (n) **	Total	29	13	42
	Breast	24	11	35
	Ovarian	1	1	2
	Other	4	1	5

* Twenty-five of the *BRCA1* mutation carriers had ER/PR/HER2 triple negative breast cancer (TNBC). None of the 23 *BRCA2* mutation carriers had TNBC.

** This represents the patients treated at our institution with chemotherapy: most breast cancer patients received four cycles of dose dense doxorubicin & cyclophosphamide followed by four cycles of paclitaxel; ovarian or peritoneal cancer patients received 4–8 cycles of carboplatin & paclitaxel (Table S1).

Table 2

NCI CTCAE grading system for hematologic toxicity

lineage	Grade 1	Grade 2	Grade 3	Grade 4
Neutrophils	<LLN to 1,500/mm^3	1,000 to 1,500/mm^3	500 to 1,000/mm^3	<500/ mm^3
Platelets	<LLN to 75,000/mm^3	50,000 to 75,000/mm^3	25,000 to 50,000/mm^3	<25,000/ mm^3
Hemoglobin	<LLN to 10 g/dL	8.0 to 10.0 g/dL	<8.0 g/dL	Life-threatening consequences
Lymphocytes (total)	<LLN to 800/mm^3	500 to 800/mm^3	200 to 500/mm^3	<200/ mm^3

Neutropenia, thrombocytopenia, anemia, and lymphopenia were determined from the complete blood count after chemotherapy and the lowest count was used for calculating grade of toxicity. All patients with sustained fever of > 100.4°F in the midst of chemotherapy-induced grade 4 neutropenia received a first course of IV antibiotics in-hospital. Taken from NCI CTCAE, version 3.0: National Cancer Institute Common Terminology Criteria for Adverse Events; LLN: lower limit of normal.