**Brca1** deficiency causes bone marrow failure and spontaneous hematologic malignancies in mice

Aparna Vasanthakumar¹, Stephen Arnovitz¹, Rafael Marquez¹, Janet Lepore¹, George Rafidi¹, Anase Asom¹, Madison Weatherly¹, Elizabeth M. Davis¹, Barbara Neistadt¹, Robert Duszynski,¹ James W. Vardiman,³ Michelle M. Le Beau,¹,² Lucy A. Godley,¹,²,⁴ and Jane E. Churpek¹,²,⁴,⁵

¹Section of Hematology/Oncology, Department of Medicine, The University of Chicago, Chicago, IL, USA, ²The University of Chicago Medicine Comprehensive Cancer Center, The University of Chicago, Chicago, IL, USA, ³Department of Pathology, The University of Chicago, Chicago, IL, USA, ⁴Center for Clinical Cancer Genetics, The University of Chicago, Chicago, IL, USA

⁵Corresponding Author:
Jane E. Churpek, MD
The University of Chicago
5841 S. Maryland Ave MC2115
Chicago, IL 60637
Phone: 773-834-1076
Fax: 773-702-9268
Email: jchurpek@bsd.uchicago.edu

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**Key Points**

- Brca1 deficiency causes Fanconi anemia-like cytopenias, mitomycin C hypersensitivity, and spontaneous bone marrow failure.

- Brca1 is critical for maintenance of normal hematopoietic progenitor function and genomic stability in the bone marrow.
Abstract
BRCA1 is critical for maintenance of genomic stability and interacts directly with several proteins that regulate hematopoietic stem cell function and are part of the Fanconi anemia (FA) double strand break DNA repair pathway. The effects of complete BRCA1 deficiency on bone marrow function are unknown. To test the hypothesis that Brca1 is essential in hematopoiesis, we developed a conditional mouse model with Mx1-Cre mediated Brca1 deletion. Mice lacking Brca1 in the bone marrow have baseline cytopenias and develop spontaneous bone marrow failure (BMF) or diverse hematologic malignancies by six months of age. Brca1/− bone marrow cells have a reduced capacity to form hematopoietic colonies in vitro and to reconstitute hematopoiesis in irradiated recipients, consistent with a hematopoietic progenitor functional defect. Brca1/− BM cells also show FA-like hypersensitivity to the DNA cross-linking agent, mitomycin C, and karyotypes feature genomic instability. Taken together, our results show that loss of Brca1 in murine bone marrow causes hematopoietic defects similar to that seen in people with FA, providing strong evidence that Brca1 is critical for normal hematopoiesis and that Brca1 is a bona fide FA-like gene.
Introduction

Fanconi Anemia (FA) is an inherited bone marrow failure (BMF) syndrome characterized by hypersensitivity to DNA cross-linking agents, congenital anomalies, BMF, and an increased risk of developing leukemia and solid tumors\(^1\). FA is caused by mutations in one of seventeen genes that make up the FA DNA repair pathway\(^2-4\).

BRCA1 binds directly to several FA proteins, all of which are essential for normal hematopoiesis, but the effects of BRCA1 deficiency on hematopoiesis are unknown. Several lines of evidence suggest that BRCA1 may also be an important regulator of hematopoiesis. First, \(BRCA1\) is highly expressed in hematopoietic tissues\(^5,6\), whereas its expression is lost in myeloid leukemias\(^7,8\). Second, overexpression of \(Brca1\) in the bone marrow (BM) disrupts stem cell quiescence and differentiation\(^9\). Lastly, the first person ever reported to have biallelic \(BRCA1\) mutations experienced unusually severe myelosuppression after exposure to the DNA crosslinking agent carboplatin\(^10\), providing direct evidence that \(BRCA1\) is important in human hematopoiesis.

To test whether \(BRCA1\) has a critical role in hematopoiesis, we created a conditional, \(Mx1-Cre\) induced \(Brca1\) deficiency mouse model. We demonstrate that mice with homozygous deficiency of \(Brca1\) in the BM have FA-like cytopenias, DNA crosslinking agent hypersensitivity, and susceptibility to BMF and hematologic malignancies (HM). Furthermore, \(Brca1^{+/}\) cells have a hematopoietic precursor functional defect characterized by a reduced capacity to form hematopoietic colonies \(in vitro\) and to reconstitute hematopoiesis in
irradiated recipient mice, supporting a critical role for Brca1 in normal BM function.

**Methods**

**Generation and monitoring of conditional Brca1 deficient mice**

All mice were housed in a pathogen-free AAALAC-accredited barrier facility maintained under an IACUC approved protocol. Mice with a floxed Brca1 allele were bred with Mx1-Cre+ mice to generate Brca1+/+, Brca1+/-, and Brca1-/- mice after poly I:C injections (Figure S1A-C). Targeted Brca1 deletion was confirmed by PCR and real-time PCR (Figure S1C-D). Mice were monitored daily for the development of tumors and with monthly tail vein bleeds to measure complete blood counts. Full necropsy was performed on any mouse with changes in appearance indicative of illness. Histopathology, flow cytometry, and spectral karyotyping (SKY) were performed on isolated tissues.

Detailed methods are included in Supplemental Data.

**Results and Discussion**

As early as one month of age, Brca1-/- mice demonstrated a macrocytic anemia (hemoglobin (Hb) levels: 10.3±3.3 versus 12.5±3 g/dL, p=0.042 (Figure 1A) and mean corpuscular volume 66.3±5.0 versus 55.4±4 fL, p<1 x 10^-5 (Figure 1B)) and had lower mean total white blood cell counts compared to Brca1+/+ mice (Figure S2).

Four of 13 (30%) Brca1-/- mice developed a steady decline in their Hb levels, eventually causing lethargy and sacrifice (Figure S3A). Necropsy
confirmed severe anemia (mean Hb 6.95±0.6 g/dL), and peripheral blood (PB) smears showed polychromasia with irregular red blood cell (RBC) morphology that was not present in Brca1+/+ mice (Figure 1C-D). BM sections were also hypocellular compared to age-matched Brca1+/+ mice (Figure 1E-F, Figure S8-S9), consistent with spontaneous BMF. Spectral karyotyping of whole BM from two BMF Brca1−/− mice (Figure 1G-H and Figure S3B-C) revealed multiple cytogenetic abnormalities, resulting from 18 and 16 double strand breaks (DSBs) detected in the 20 cells examined in each mouse, indicative of genomic instability.

By day 140-190, six additional Brca1−/− mice (6 of 13; 50%) developed HM, including four lymphomas, one acute myeloid leukemia (AML), and one erythroleukemia (Figure 2A-B). Trp53 sequencing identified DNA binding domain mutations in two of the lymphomas (Figure 2B). Histologic review of tissues from these mice demonstrated malignant cells circulating in the PB, disrupting hematopoietic organ architecture, and infiltrating into non-hematopoietic organs (Figure 2C-J, Figure S9). Splenic cells isolated from the mouse with erythroleukemia were transplanted into two sub-lethally irradiated recipient mice, both of which developed erythroleukemia. Spectral karyotyping of malignant cells from the secondary transplant revealed multiple chromosomal translocations, a result of DSBs (Figure 2K-L), indicative of genomic instability in a malignancy in this model as well. None of the Brca1+/+ or Brca1+/− mice developed a malignancy during 12 months of follow-up. These results demonstrate that lack of Brca1 within the BM leads to PB cytopenias,
macrocytosis, spontaneous BMF, and susceptibility to HM reminiscent of the hematopoietic defects observed in human FA\textsuperscript{14,15}.

To investigate the etiology of these hematopoietic defects, we performed flow cytometry on single cell suspensions from BM and spleen to assess for differences in specific hematopoietic cell populations. In the spleens of BMF mice, we found an accumulation of early RBC precursors at the basophilic erythroblast (CD71+, Ter119+) stage relative to \textit{Brca1}\textsuperscript{+/+} mice (\textit{p}=0.003) (Figure 1I-J) that was not present in their BM (Figure S3D), suggesting a niche effect.

We then assessed for differences in cell populations in the BM of two month old \textit{Brca1}\textsuperscript{-/-} mice, prior to the expected age of BMF or HM development, versus age-matched \textit{Brca1}\textsuperscript{+/+} or \textit{Brca1}\textsuperscript{+/+} mice. These experiments demonstrated an expansion of Lineage-, Sca-1+, c-Kit+ (LSK) cells, a population enriched for hematopoietic stem and progenitor cells (HSPCs) (Figure S5), but no differences in the numbers of LT-HSC, ST-HSC, MPPs, or other committed progenitors (Figures S5-S7).

Because most other single gene FA mouse models demonstrate reduced HSPC numbers and function,\textsuperscript{16,17} we tested the hematopoietic progenitor function of \textit{Brca1}\textsuperscript{-/-} BM cells. Using methylcellulose colony forming assays, we found that \textit{Brca1}\textsuperscript{-/-} BM cells formed fewer hematopoietic colonies \textit{in vitro} (44.4±31.9) than \textit{Brca1}\textsuperscript{+/+} cells (200.3±30.5, \textit{p}=0.004). Similarly, BM cells from two-month old \textit{Brca1}\textsuperscript{-/-} mice were unable to reconstitute hematopoiesis in lethally irradiated congenic mice (Figure S4). These data demonstrate that although LSK numbers
are expanded, HSPCs in our model have decreased function as seen in other FA models.

Finally, to test $Brca1^{-/-}$ BM cells for hypersensitivity to DNA cross-linking agents, a classic hallmark of FA, we performed colony forming assays in the presence of increasing concentrations of mitomycin C (MMC). $Brca1^{-/-}$ BM cells demonstrated increased sensitivity compared to $Brca1^{+/+}$ cells at all MMC concentrations tested (Figure 1K). Taken together, these data demonstrate that Brca1 is critical for normal hematopoiesis and maintenance of genomic stability in the BM, and its deficiency results in an FA-like hematopoietic phenotype in mice.

Until recently, $BRCA1$ was not considered a true member of the FA pathway because the effects of homozygous deficiency of this gene in people were not known$^3$. Recently, two patients with biallelic $BRCA1$ mutations, each with one hypomorphic and one null allele, were reported. The FA-like phenotype in these patients included developmental anomalies and increased sensitivity of lymphocytes to chromosomal breakage with exposure to diepoxybutane$^4,10$, leading to the designation of $BRCA1$ as the seventeenth FA gene, $FANCS$. However, neither of these individuals has the characteristic FA BMF phenotype, leaving the effects of $BRCA1$ deficiency on BM function unknown.

Our data provide direct evidence of a FA-like hematopoietic defect in $Brca1$ deficient BM, strengthening the evidence that $BRCA1$ is a bona fide FA-like gene with a critical role in hematopoietic function. Furthermore, these mice
provide a model system in which to determine the effects of Brca1 haploinsufficiency, as seen in humans, on BM function, as well as ready access to a renewable source of \textit{Brca1} deficient tissue for characterization of Brca1’s complex functions. Lastly, as nearly all other single FA gene mouse models have not recapitulated the BM phenotype of FA\textsuperscript{16,18}, further investigation of our model may provide novel insights into FA pathogenesis.

\textbf{Authorship}

AV, MML, LAG, and JEC designed the research; AV, SA, RM, JL, GR, AA, MW, RD, ED performed research; AV, SA, RM, GR, BN, ED, MML, JWV, LAG, and JEC analyzed data; AV and JEC wrote the manuscript. All authors edited and approved the final manuscript.

\textbf{Conflicts of Interest}

The authors have no relevant conflicts of interest.

\textbf{Acknowledgements}

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References


Figure Legends

Figure 1. *Brca1* deficiency causes peripheral blood cytopenias, BM failure featuring genomic instability and DNA cross-linking agent hypersensitivity.

Complete blood counts from *Brca1*+/+ (red), *Brca1*+/− (green), and *Brca1*−/− (blue) mice were measured monthly up to 6 months of age. (A) Hemoglobin (Hb) concentration (g/dL); (B) Mean corpuscular volume (MCV) (fL). The number of mice in each cohort at each time point analyzed is listed in Supplemental Figure S2. ANOVA was used to analyze for differences in counts at each time point (* P < 0.01). Peripheral blood smears from a (C) *Brca1*+/+ (7145) and a (D) *Brca1*−/− (7386) mouse are shown (10X magnification). BM sections from a (E) *Brca1*+/+ (7145) and a (F) *Brca1*−/− (7386) mouse are shown (10X magnification). (G-H) Spectral karyotyping analysis revealed multiple structural abnormalities including chromatid exchanges and premature centromere divisions. Representative cell karyotype in G: 40,XX,chte(2;5)(F1;C2),chte(9;12)(F1;E),pcd(16)(A)[1]. Abbreviations: chte=chromatid exchange, pcd=premature centromere division, chtb=chromatid break, chtg=chromatid gap, chrb=chromosome break, chrq=chromosome gap. (I) Representative FACS plots from spleen cells from a *Brca1*+/+ (B7292) and a *Brca1*−/− (B7221) mouse stained with antibodies against CD71 and Ter119. (J) Average proportion of spleen cells accumulating in regions I, II, III, and IV of red blood cell differentiation after staining with antibodies against CD71 and Ter119. Student’s *t*-test was used to analyze the differences in the proportion of cells within each region. (K) Sensitivity of *Brca1*−/− cells (blue)
relative to $Brca1^{+/+}$ cells (red) in methylcellulose colony forming assays to mitomycin C at doses of 0, 5, 10, 20, and 50 nM. Averages are shown with SEM.

Figure 2. $Brca1$ deficiency increases susceptibility of mice to hematologic malignancies characterized by leukemic infiltration of multiple organs, consistent with the Bethesda criteria $^{19,20}$. (A) Cumulative disease incidence curves for $Brca1^{+/+}$ (dash-dot-dot line), $Brca1^{+/-}$ (dashed line), and $Brca1^{-/-}$ (solid line) mice. Statistical significance was calculated using the log-rank test. The number of mice in each cohort at each time point analyzed is listed in Supplemental Figure S9H. (B) Characteristics of diseased mice and specific hematologic malignancy diagnosis. Presence or absence of a $Trp53$ mutation in each tumor is indicated. (C-F) Organs isolated from 7167, a $Brca1^{-/-}$ mouse that developed a T-cell lymphoma. (C) Peripheral blood smear stained with Wright-Giemsa showing the presence of lymphoma cells (10X magnification). (D) Peripheral blood flow cytometry analysis using antibodies against T-lymphoid markers CD4 and CD8, showing the malignant cells to be CD4+/CD8+. (E) BM stained with H&E, showing extensive involvement with lymphoma (10X magnification). (F) Thymus stained with H&E, showing effacement of the normal thymic architecture by infiltrating lymphoma cells (50X magnification). (G-J) Organs isolated from B7258, a $Brca1^{-/-}$ mouse that developed an erythroleukemia. (G) Peripheral blood smear stained with Wright-Giemsa, showing many erythroid blasts (50X magnification). (H) Peripheral blood flow cytometry analysis using antibodies against CD71 and Ter119. (I) BM stained
with H&E, showing extensive leukemic involvement (10X magnification). (J) Liver stained with H&E, showing extensive infiltration by leukemic cells (50X magnification). (K) Spectral karyotyping analysis of erythroleukemia cells from a secondary transplant recipient mouse revealed an abnormal clone characterized by structural rearrangements: Karyotype: 40,XX,t(1;15)(D;E), t(12;17)(D3;E2), and del(14)(D3E3). (L) Cytogenetic abnormalities identified within the erythroleukemia. Abbreviations: del=deletion, idem=“the same” as the stemline clone listed first.
Cytogenetic abnormalities

40,XX[13]
40,XX,chrb(4)(C2)[1]
40,XX,chtb(2)(H1),chtg(6)(B1)[1]
40,XX,chtb(1)(H5),chrg(5)(D)[1]
40,XX,chrg(2)(E2)[1]
39,XX,chtb(2)(B),chrb(3)(F1),chrg(13)(C3),chrg(15)(E),-16,chtb(17)(B)[1]
40,XX,chte(2;5)(F1;C2),chte(9;12)(F1;E),pcd(16)(A)[1]
40,XX,t(1;17)(H4;A2)[1]

Figure 1

H

A

B

C 7145 (Brca1+/+)
D 7386 (Brca1+/-)

E 7145 (Brca1+/-)
F 7386 (Brca1-/-)

G

H

I

J

K

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Cytogenetic abnormalities

40,XX,t(1;15)(D;E),t(12;17)(D3;E2),del(14)(D3E3)[9]/
40,idem,t(1;9)(D;D),t(12;17)(D3;E2),del(14)(D3E3),t(14;18)(E3;E3)[1]/
40,idem,t(6;7)(D;F3)[1]/
#40,XX,t(1;9)(D;D),t(12;17)(D3E2),del(14)(D3E3),t(14;18)(E3;E3)[1]/
40,idem,t(1;11)(F;E1)[1]/
40,idem,t(2;7)(H3;C)[1]/
40,idem,t(16;17)(B3;E2)[1]/
40,idem,t(1;11)(F;E1)[1]/

Figure 2

A

B

C

D

E

F

G

H

Cytogenetic abnormalities

40,XX,t(1;15)(D;E),t(12;17)(D3;E2),del(14)(D3E3)[9]/
40,idem,t(9;11)(A1;D)[1]/
40,idem,t(2;7)(H3;C)[1]/
40,idem,t(16;17)(B3;E2)[1]/
40,idem,t(1;11)(F;E1)[1]/
40,XX,t(1;9)(D;D),t(12;17)(D3E2),del(14)(D3E3),t(14;18)(E3;E3)[1]/
40,idem,t(6;7)(D;F3)[1]/
40,idem,t(8;17)(C3;B)[1]/
40,idem,t(10;12)(B2;E)[1]
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