# CONSEQUENCES OF *BRCA1* EPIGENETIC SILENCING ON HOMOLOGOUS RECOMBINATION AND DISEASE PROGRESSION IN MYELOID NEOPLASMS

by

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**Abstract**

Myeloid malignancies are hematological disorders encompassing chronic myeloid neoplasms to acute leukemias. In recent years, insight from genome-wide discovery studies has improved our understanding and ability to predict prognosis and treatment outcome. Despite these advances, there remains considerable clinical heterogeneity within current classification systems as these diseases often present with diverse and overlapping pathological features. At the same time, there has been limited success in translating these findings into effective therapeutics to improve overall survival. Increasingly, it is recognized that these complex and dynamic molecular changes converge into a small number of biological pathways. Therefore, a pathway-driven approach identifying commonly perturbed processes could yield greater success in developing broadly applicable therapeutics. One promising candidate is the homologous recombination (HR) pathway responsible for repairing double-stranded breaks, since most myeloid neoplasms are characterized by gross chromosomal instability. To objectively assess HR repair in fresh mononuclear cells from myeloid malignancy patients, we developed an *ex vivo*, short-term assay that determines HR repair based on nuclear RAD51 foci induction after DNA damage. Using this technique, we observed HR defects in 9 of 21 myeloid malignancy samples. Since there is little evidence for mutational alterations in HR genes, we screened HR gene promoters and observed *BRCA1* promoter methylation in a significant subpopulation (22/96 samples) that

strongly associates with disrupted HR repair. To our knowledge, this is the first report linking *BRCA1* methylation to HR defects in patient samples. Next, we validated the patient samples findings by silencing *BRCA1* expression in AML cells that recapitulated the HR defects. We treated AML cells with poly(ADP-ribose) polymerase (PARP) inhibitors and observed increased sensitivity with *BRCA1* repression, providing a mechanistic justification for previous studies highlighting toxicities with PARP inhibitors in myeloid malignancies. The high prevalence of *BRCA1* silencing led us to consider additional roles in driving myeloid disease, as it is known to repress microRNA-155 (miR-155) that is frequently elevated in myeloid malignancies. By correlating gene expression in the patient samples, we found an inverse correlation between *BRCA1* and *miR-155* levels. miR-155 is frequently elevated in myeloid malignancies and its targets include key regulators of inflammation (SHIP1) and myeloid differentiation (PU.1). Our results here show that *BRCA1* loss due to promoter methylation not only contributes to HR defects, but also contributes to disease progression via miR-155 upregulation.

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# Chapter 1: Introduction

## Heterogeneity of Myeloid Neoplasms Impacts Clinical Management

Myeloid neoplasms are clonal hematological disorders arising from malignant myeloid progenitor stem cells that present with diverse and overlapping clinicopathological features. In 2008, the World Health Organization (WHO) categorized myeloid malignancies into five major groups based on morphologic, genetic and clinical features: (1) acute myeloid leukemia (AML) and related precursor neoplasms; (2) myelodysplastic syndromes (MDS); (3) myeloproliferative neoplasms (MPN); (4) overlap MDS/MPN; and (5) myeloid neoplasms associated with eosinophilia and abnormalities of growth factor receptors.[1] However, significant clinical heterogeneity remains and it is acknowledged that myeloid disorders represent closely linked entities along a continuum, and chronic MPNs, MDS and MDS/MPN have varied propensities of transformation to acute leukemias.[2] The importance of defining molecular lesions driving myeloid malignancies that can lead to novel therapies is underlined by the fact that the majority of these conditions affect individuals above 60 years, creating significant challenges with current treatments such as intensive chemotherapy and bone marrow transplantation.[3,4] Even with high-dose induction therapy, most patients have significant disease mortality with shorter remissions and a dismal 3-year survival of 15%.[5,6] Despite promising successes, it is well accepted that most therapies are incapable of completely eradicating the malignant clone and current treatment is limited to supportive care without improving overall survival.[7,8]

To better define the molecular lesions driving myeloid malignancies, genome-wide discovery studies have revealed a wide spectrum of biologically significant somatic alterations that has provided insights into the pathogenesis of myeloid diseases.[9-11] The identification of the BCR-ABL fusion gene in chronic myelogenous leukemia (CML) samples led to the development of ATP-competitive inhibitors targeting to the catalytic activity of the ABL kinase, and treatment with these inhibitors results in impressive clinical outcomes that allowed patients to have normal life expectancies.[12-15] The use of all-trans retinoic acid (ATRA) to convert acute promyelocytic leukemia (APL) into a disease with long-term remissions can be attributed to the characterization of distinct chromosomal translocations involving the retinoic receptor alpha (RARA).[16]

However, there remain significant challenges in our understanding of the biological relevance of the over 500 disease alleles discovered by whole-genome approaches.[9,10] Development of cost-effective assays capable of detecting the wide spectrum of somatic alterations to deliver clinically actionable information will also face considerable obstacles. While somatic mutations in the Janus kinase 2 (*JAK2*), thrombopoietin receptor (*MPL*) and Calreticulin (*CALR*) genes are useful in diagnosing BCR-ABL negative MPNs, these alterations are present in all MPN subtypes and do not account for the differences in prognosis among polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF).[17,18] As we continue to improve our understanding of mutational profiles, a function-based classification of cancer-associated changes would allow researchers to further decipher the process of malignant transformation and progression.

**Designing Novel Therapeutics to Target Shared Pathway Deficiencies**

One solution to understand the complex genetic landscape is to characterize disrupted pathways in lieu of individual genomic lesions, given that recurrent mutations impact a restricted number of biological pathways such as RNA splicing, inflammation, epigenome regulation and DNA damage repair.[9] With regards to MPNs, mutations in *JAK2*, *MPL* and *CALR* share similar transcriptional profiles in activating the JAK/STAT pathway, leading to the development of inhibitors targeting JAK signaling.[7,19] Treatment with JAK inhibitors results in a significant reduction in splenomegaly and substantive symptomatic relief.[8] Likewise, mutations in DNA methylation modifiers *DNMT3A* and *TET2* found in a wide range of myeloid neoplasms have driven development of therapies that revert the epigenome to a normal state, exemplified by the FDA approval of azacitidine, a DNA methyltransferase (DNMT) inhibitor, for the treatment of MDS.[20] Azacitidine and decitabine, its deoxy derivative, are cytidine analogs that incorporate into DNA and bind irreversibly to DNMTs in order to inhibit methylation.[21] Clinical trials with these drugs produce complete cytogenetic remissions and improved survival compared to traditional agents such as cytarabine that are observed even across the diverse manifestations of MDS.[22] These encouraging developments lend weight to the approach of targeting novel disease-relevant pathways to reverse disease progression and improve patient stratification in myeloid malignancies.

**Targeting Defects in Homologous Recombination Repair**

It is widely accepted that most cancers exhibit genetic instabilities in the form of unbalanced translocations, gains and losses of large sections of chromosomes.[23] The interest in studying cytogenetic aberrations in myeloid diseases can be traced back to the discovery of the BCR-ABL translocation in CML, and subsequent studies have since reported chromosome abnormalities that increase in frequency with disease progression.[24-26] Interestingly, many recurrent cytogenetic aberrations known to impact disease biology have been reported across diverse clonal myeloid diseases (MPNs, MDS/MPNs, sAMLs).[27,28] In a significant proportion of AML and MDS, chromosomal instability has been attributed to the aberrant repair of double-strand DNA breaks (DSBs).[26] DSBs arise with increased frequency in tumor cells from reactive oxidative stress generated by oncogenes, and can also result from external insults such as chemotherapy.[29] If these lesions are not resolved in a timely fashion, they can be detrimental on genomic integrity and lead to chromosomal translocations, cell death and oncogenesis.[30]

In order to maintain genomic integrity, the main DSB repair mechanisms are the non-homologous end joining (NHEJ) or homologous recombination (HR) pathways.[31] Upon induction of DSBs, a coordinated response is initiated beginning with the binding of ataxia telangiectasia mutated (ATM), ATM-related (ATR) and DNA-dependent protein kinases that phosphorylate histone H2AX variant near damage sites that in turn recruits downstream repair complexes.[32] NHEJ is the preferred mechanism of DSB repair in $G_1$ phase and is initiated when Ku70 and Ku80 form heterodimers that bind the free

ends at sites of damage. After further processing, the DNA ends are then ligated by DNA ligase IV-XRCC4.[33] This process is known to generate errors in the form of gain or loss of nucleotides <20bp that contribute to genomic instability.[34] In contrast, HR relies on homologous sequences on a sister chromatid during S phase for error-free repair.[35]After end resection by the MRE11/RAD50/NBS1 complex, RAD51 monomers bind to the single-stranded DNA ends to initiate homology search, strand invasion and DNA synthesis. The ends are then ligated before resolving the Holliday junctions.[30] Given the deleterious effects of DSB lesions, genetic alterations in components of the HR pathway have been implicated in a variety of malignancies.[31,36] Inherited conditions due to mutations in *ATM*, *NBS*, *DNA ligase IV* have increased predisposition to cancers including leukemias.[31,37,38] Germ-line defects in *BRCA1* and *BRCA2* are found in the familial breast cancers that have a two-fold increase in gross chromosomal changes compared to sporadic cancers.[39,40] In addition, Fanconi anemia and Bloom's syndrome patients exhibiting pronounced HR defects have elevated risk of developing hematological malignancies.[41] Mechanistically, embryonic lethality in mouse knockout models for HR components is often preceded by gross chromosomal rearrangements.[31,42] Consequently, the ability to characterize this phenotype has important implications not only in improving our understanding of disease pathobiology, but to allow the rational design of therapies targeting HR defects in tumor cells.

Increasingly, the goal of anticancer agents is moving from high-dose, non-specific cytotoxic agents to individualized and highly selective therapy specific to neoplastic cells. In this regard, the concept of synthetic lethality has generated great interest by

exploiting cancer-specific defects to increase the therapeutic index between cancer and normal cells, making it possible to design treatments against previously undruggable targets while minimizing side effects. Base excision repair (BER) is an appealing target since disruption of this pathway leads to the accumulation of single-stranded breaks and subsequent stalled replication forks.[43-45] While these stalled forks are repaired in normal cells with intact HR, these lesions persist in HR defective cancer cells that result in acute cell death.[43-45] Poly(ADP-ribose) polymerase 1 (PARP1) localizes to DNA nicks via its N-terminal double zinc finger DNA-binding domains and catalyzes the covalent attachment of ADP-ribose groups using nicotinamide adenine dinucleotide (NAD+) as a substrate. [46] This process of PARylation alters the local chromatin environment to recruit other BER components for efficient DNA repair. PARP1 is also associated with nucleotide excision repair and the repair of stalled replication forks.[47] Given the plethora of repair processes for PARP1, increased genetic instability and hypersensitivity to DNA damaging agents and radiation have been observed in *PARP1-/-* null mice. [48] On the other hand, PARP1 overexpression has been implicated in resistance to chemotherapy or radiotherapy.[49] Given the above observations, it is probable that PARP1 inhibition in HR defective cells will have enhanced toxicity since the resulting DSBs cannot be repaired appropriately. In 2005, two groups demonstrated increased susceptibility to PARP1 inhibition of *BRCA1*- and *BRCA2*-deficient systems compared to cells with normal BRCA functions.[43,50] These results have since accelerated the development of PARP inhibitors for treating multiple solid and hematologic malignancies. In December 2014, the first PARP inhibitor (olaparib) was approved for heavily pre-treated advanced ovarian cancers with germ-line BRCA mutations after showing durable objective responses,

further signifying the immense clinical significance of this concept. Since most patients with myeloid malignancies are advanced in age and cannot tolerate intensive chemotherapy, such synthetic lethal treatments offer great potential.

Given the complex network of proteins involved in HR, the function of this pathway can be compromised by alterations in one of more genes that are not limited to *BRCA1* and *BRCA2*. Indeed, subsequent studies have reported that defects in RAD51, MRN, ATM, and ATR, also sensitize cancer cells to PARP1 inhibition.[51,52] Large-scale loss-of-function screens also revealed genes involved in other process that mediated toxicity to PARP inhibition such as *DDB1*, *XAB2* in nucleotide excision repair, and *PTEN*, a negative regulator of PI3K.[53,54] Aside from mutations, epigenetic silencing of *BRCA1* has also been shown to increase sensitivity to PARP inhibition in cells and xenografts.[55] In chronic myelogenous leukemia (CML), the BCR-ABL fusion protein was shown to down-regulate BRCA1 on a post-transcriptional level that disrupts proficient DNA repair after ionizing radiation.[56] Tumor-associated hypoxia can also potentiate the efficacy of PARP inhibitors since it is known to repress expression of HR genes to attenuate the DNA-damage response.[57,58] Consequently, the range of diseases amenable to PARP inhibitors must be expanded beyond the current model of *BRCA* mutated breast and ovarian cancers. To identify these tumor types and realize the full potential of this synthetic lethal approach, assays based on novel, reliable biomarkers are critical in order to identify HR defects in patient tissue biopsies.

**Functional Assays for Homologous Recombination Repair**

With immense interest in identifying cancers with HR defects, multiple studies have presented methods using assays for gene expression, DNA methylation status and comparative genome hybridization.[59-61] Other groups have examined HR repair by measuring persistence of phosphorylated histone variant H2AX foci that forms at DSBs.[62] These platforms have variable degrees of predictive power but are not direct measures of HR repair. Initial assays for direct assessment of HR involve induction of DSBs using *I-SceI* enzyme in cells stably expressing GFP reporter plasmids, however it is not a feasible approach in primary samples given the challenges with transfection and *ex vivo* culture.[63] Since RAD51 plays a central role in HR by catalyzing strand invasion into the homologous strand, RAD51 foci formation following DNA damage offers a physiologically relevant readout of HR function.[64] Consequently, lack of RAD51 foci induction is well established as a marker of HR-defective *BRCA1/2* mutated cells and multiple cancers.[55,65-67]

Hematological malignancies offer readily accessible transformed cells for *ex vivo* evaluation of HR repair, and several studies have exploited this fact to show defects in a small number of MDS and MDS/AML primary samples.[34,67,68] However, these procedures require extended *ex vivo* (24h) treatment of primary myeloid samples with PARP inhibitors that not only introduces significant stress to malignant cells, but also are subject to confounding factors such as drug uptake and metabolism. Consequently, further improvements are needed to generate robust results and minimize sample loss during processing.

Ionizing radiation is an alternative approach that rapidly introduces DSBs to monitor HR repair.[69] After γ-radiation exposure with caesium-137, cells are allowed to resolve DSBs for 2-6hrs, before fixing and staining for distinct γH2AX and RAD51 nuclear foci as markers of DNA damage and HR repair respectively. Measurements by immunofluorescence indicate a rapid induction (<30min) in γH2AX foci that decreases as DSBs are repaired. Concurrently, RAD51 foci peak at 6h post-radiation before decreasing to baseline levels.[69] This technique allows significantly shortening of *ex vivo* treatment, and has been validated as a reliable marker of PARP inhibitor sensitivity in primary breast and ovarian cancer cultures.[65,70-72] Given the evidence suggestive of HR defects in myeloid malignancies, a direct measure of HR activity in this disease would be informative.

**Epigenetic Changes in Myeloid Malignancies on Homologous Recombination**

To identify key driver mutations amongst the over 400,000 gene mutations recorded in the Catalogue of Somatic Mutations in Cancer (COSMIC) database, Vogelstein *et al.* proposed a criteria based on mutation patterns and reported a total of 294,881 mutations in 125 genes that confer a growth advantage to the malignant clone.[73] Intriguingly, nearly half of these genes are involved in direct regulation of DNA methylation and histone modification, highlighting the central role of epigenetic dysregulation in tumorigenesis. Epigenetic regulation plays a role in establishing tissue-specific cellular identities, and refers to heritable gene expression changes not due to gene sequence changes.[74,75] Cancer cells frequently exhibit epigenome alterations

including genome-wide losses of DNA methylation with localized increases at CpG islands in promoter regions, as well as chromatin structure changes that regulate nucleosome packaging.[75,76] Collectively, these changes have been proposed as key players in cancer initiation and progression.[75,77]

As stated above, the epigenome can be regulated by modifications to the histones and DNA bases in the genome. These changes affect the configuration of nucleosomes, which are the basic units of chromatin that each consists of DNA wrapped around a core comprising two copies of the H2A, H2B, H3 and H4 variants.[78] In turn, the chromatin structure around the transcription start site is a critical determinant of the transcription state of the corresponding gene.

For actively transcribed genes, nucleosomes are widely spaced with a nucleosome-free region at the transcription start site to facilitate access to components of the gene transcription machinery. On the other hand, nucleosomes are arranged in a compact fashion and regularly spaced for transcriptionally silenced genes.[75-77] Also, key amino acid residues on histone tails that protrude from nucleosomes can be modified by the addition/removal of methyl or acetyl groups that alters electrostatic charges or acts as protein binding domains. [75-77,79] Since the predominant acetylation occurs at lysine residues, acetylation alters the charge of the histone tail from positive to neutral, thereby reducing the affinity of histones to the negatively charged phosphate backbone of DNA. [75-77,79] Nucleosome formation may also be enhanced by positive charges of H4 histones and negative charges on the surfaces of H2A histone fold domains. [75-77,79] Disruption of

HDAC function and altered expression has been shown in multiple cancers, in which HDACs or histone methyltransferases (HMTs) form oncogenic fusion proteins and are aberrantly recruited to gene promoters.[80] For example, the t(8;21) translocation in AML generates the AML1-ETO fusion protein that recruits HDACs to repress AML1 target genes and block myeloid differentiation.[80] Likewise, in acute promyelocytic leukemia (APL), the PML-RARα fusion results in binding of HDACs and DNMTs to silence RARα target genes.[81] Histone modifiers can also cause gene up-regulation, as observed with fusion proteins involving MLL, a H3K4 methyltransferase, through induction of H3K4me3 and H3K79me2.[82] Understandably, there has been interest in developing HDAC inhibitors that have shown anticancer effects including cell death, cell cycle arrest, senescence, differentiation and autophagy.[83]

In addition to histone modifications, the epigenome can be altered by DNMTs that attach a methyl group from S-adenosyl-methionine (SAM) onto the 5-carbon position of cytosine base to form methyl-cytosine. This tends to occur predominantly at CpG dinucleotides, of which over 70% are methylated in the human genome in normal cells. In contrast, "CpG islands" that are in close proximity to promoter regions of over half of the genes in the human genome are highly concentrated in unmethylated CpGs.[84] These regions of at least 200bp have an observed to expected CpG ratio greater than 60%and a GC percentage greater than 50%.[84] Loss of DNA methylation at repetitive regions of the genome may result in the expression of inserted viral genes and repeat elements, or disrupt the functional stability of pericentromeric regions.[75] On the other hand, methylation of CpG islands results in the aberrant silencing of tumor suppressor

genes in cancer cells and can represent the second hit of the Knudson two-hit hypothesis to inactivate gene expression similar to gene mutations. Increasingly, it is acknowledged that the number of genes inactivated by epigenetic silencing is at least as prevalent as those attributed to mutations in cancers.[75,85,86] The biologic effects of promoter methylation are also similar to exome mutations, as seen in breast cancers where genetic and epigenetic changes in *BRCA1* produce similar gene expression microarray profiles.[75,85,87] Promoter hypermethylation of *MLH1* also generates microsatellite instability in colon tumors analogous to mutational inactivation. As mentioned above, DNMTs can be aberrantly recruited to CpG islands near gene promoters to inactivate tumor suppressors by further binding of other transcription repressor proteins. In contrast to gene mutations, DNA methylation can be reversed by demethylating agents such as azacitidine to re-express silenced genes. Furthermore, DNA hypermethylation changes are known to occur early in cancer progression, hence there is the potential for cancer prevention by inhibiting or reversing these alterations.[77,88]. This strategy has shown promising results in MDS patients where reactivation of the cyclin-dependent kinase inhibitor p15 was observed with azacitidine treatment.[89] Because epigenetic changes are dynamic, aberrant promoter methylation and gene silencing can reappear once the demethylating treatment is halted, suggesting the need for sustained drug dosing for cancer therapy.[90]

The clinical implications of studying DNA methylation can be extended to its utility as a molecular biomarker for cancers. When collecting clinical material from patients, delays in processing are common that might compromise sample quality. Hence

promoter methylation determination can be more suitable for clinical applications given the stability of DNA relative to RNA and protein. In addition, detection of a single region near gene promoters for DNA methylation offers a simple approach compared to mutations in tumor suppressor genes that tend to be located throughout the gene body.[73,75] Identification of a positive methylation signal on a background of unmethylated normal samples also improves the ease of detection especially when coupled with sensitive assays such as polymerase chain reaction (PCR) methods.[75,86,91] The methylation specific PCR (MSP) technique has been successfully used to identify abnormal promoter methylation in a variety of samples (tissue, serum, sputum, urine).[75] Since DNA methylation changes are early events in cancer progression, it is possible to identify cancer from sputum using MSP analysis before clinical detection of lung tumors in smokers.[92] For myeloid malignancies such as MPN, MDS and mixed MDS/MPN, we hypothesize that studying these early epigenetic changes can shed light on pathways that can be targeted early to halt leukemic transformation.

**Objectives**

We have previously performed single nucleotide polymorphism array (SNP-A) analysis in MPNs and found chromosome losses encompassing HR genes that also correlated with defective induction of RAD51 foci.[93] In the same study, we also demonstrated decreased colony formation in samples with defective HR when treated with PARP inhibitors. Collectively, these results not only suggest that defective HR repair occurs early in myeloid malignancies, but also can be a useful biomarker for PARPi therapy to prevent disease progression. At the same time, given the widespread

observation of chromosomal instability, we hypothesize that this pathway defect can be extended to other myeloid malignancies (MDS, MDS/MPN, AML). To do so, we would need to develop a short-term *ex vivo* assay capable of assessing HR repair in primary patient samples. Our next goal is to investigate the molecular mechanisms that underlie the pronounced DNA repair defects in myeloid malignancies. Since sequencing analysis of HR repair genes in AML and MDS patients with impaired DSB repair revealed a limited number of HR gene mutations that do not fully account for the prevalence of defective HR, we investigated if epigenetic silencing via promoter methylation can account for this deficiency.[67,88,94] Finally, we are interested in understanding alternative mechanisms in which the molecular aberration drives myeloid disease progression.

## Chapter 2: *BRCA1* Promoter Methylation is Associated with Homologous Recombination Repair Defects and Elevated *miR-155* in Myeloid Malignancies

### Introduction

Myeloid malignancies are a heterogeneous collection of clonal hematological disorders that span precursor neoplasms such as chronic myeloproliferative neoplasms (MPN), myelodysplastic syndromes (MDS) and overlap mixed MDS/MPNs, to de novo acute leukemias (AML) and secondary AMLs (sAML).[1] The majority of pre-leukemic conditions (MPN, MDS, mixed MDS/MPN) affect individuals above 60 years, making potential curative treatments such as intensive chemotherapy and bone marrow transplantation difficult.[4] With the exception of BCR-ABL chronic myelogenous leukemia (CML), many of these disorders continue to be distinguished by morphological features and quantitative differences in myeloid components, reflecting the lack of molecular insight into disease-specific pathogenesis. This highlights the urgent need to elucidate novel disease pathways targetable with currently available drugs, without which patients continue to be at significant risk of bone marrow failure and leukemic transformation.[95] Given the common morphologic, cytogenetic and genetic abnormalities,[27,28] we hypothesized that the same pathways are disrupted across the diverse spectrum of myeloid neoplasms. Consequently, therapies designed to target these shared functional defects would be more efficacious for this diverse group of diseases.

However, direct analysis of the high fidelity, error-free HR pathway in myeloid malignancies is lacking compared to breast and ovarian cancers.[43] This dearth of

knowledge has therapeutic importance, given the increased interest in HR repair with the approval of olaparib, a poly(ADP-ribose) polymerase inhibitor (PARPi), for *BRCA1*-mutated ovarian cancer.[96] Since PARPi is exquisitely toxic to HR defective malignant cells and spares normal tissues, a comprehensive study of HR repair and associated molecular aberrations in myeloid malignancies could determine if patients are potentially sensitive to this therapy.[43,44] To address this gap, we developed a short-term *ex vivo* radiation-based HR assay for primary cells from myeloid neoplasms and discovered common functional defects in HR repair. Next, we investigated epigenetic silencing of HR genes to elucidate the underlying molecular events and observed frequent *BRCA1* promoter methylation that was strongly associated with HR defects. With the observation of increased PARPi sensitivity in *BRCA1*-repressed cells, we reasoned that a substantial proportion of myeloid neoplasm patients might be amenable to this form of synthetic lethal therapy, using *BRCA1* promoter methylation as an epigenetic biomarker for responders. The increased prevalence of BRCA1 silencing relative to other HR genes also suggests additional roles of BRCA1 loss in driving myeloid disease progression and here we present evidence that *BRCA1* loss up-regulates the oncomiR *miR-155* that targets multiple proteins essential for myeloid differentiation and inflammation.[97-101] In conclusion, this is the first study demonstrating that epigenetic silencing of *BRCA1* not only associates with HR defects in primary samples, but also increases miR-155 expression to accelerate myeloid transformation.

## Materials and Methods

### Patient Samples

Peripheral blood or bone marrow aspirates were collected and analyzed under IRB approved protocols at the Johns Hopkins Hospital. All patients provided informed consent in accordance with the Declaration of Helsinki. Patient characteristics are provided in Table 1. Mononuclear leukocytes were isolated by density centrifugation with Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ, USA). The ZR-Duet DNA/RNA MiniPrep kit (Zymo Research, Orange, CA, USA) was used to obtain genomic DNA (gDNA) and RNA. Samples from earlier studies were used for the initial screening of HR gene methylation.[102,103]

### HR repair assay

The HR status of primary mononuclear cells was evaluated as described by Patel *et al.* with modifications.[104] After density centrifugation, at least five million cells were re-suspended in RPMI media with 10% (v/v) fetal bovine serum (FBS), radiated with 10Gy using a Gammacell 1000A $^{137}$Cs source (Atomic Energy of Canada) at a rate of 2.3Gy/min and placed in $37^0$C, 5% $CO_2$ in humidified atmosphere for six hours. The cells were pelleted, washed with PBS and fixed with 2% formaldehyde. 250,000 cells were cytospun onto poly-lysine coated slides, permeabilized with 0.25% (v/v) Triton X-100 in PBS and incubated in blocking buffer for 1h (PBS with 1% (v/v) glycerol, 0.1% (w/v) fish skin gelatin, 0.1% (w/v) BSA, 5% (v/v) goat serum and 0.4% (w/v) sodium azide). Slides were incubated overnight at $4^0$C with RAD51 rabbit polyclonal (ActiveMotif) and

phosphor-Ser139 H2AX mouse monoclonal (Millipore) antibodies diluted 1:500 in blocking buffer. Cells were then incubated for 1h with secondary Alexa Fluor 488 goat anti-mouse and Alexa Fluor 555 goat anti-rabbit antibodies (Invitrogen) diluted 1:1000. Slides were washed, counterstained with 1μg/ml Hoechst 33258 for 5min and then mounted with Prolong Gold antifade reagent (Life Technologies).

Confocal images were obtained with a Nikon C1si confocal laser-scanning microscope with a 100X.1.4 N.A. oil-immersion objective at excitation wavelengths of 408, 488, and 561nm. Maximum projection images were generated from optical sections of 0.5μm increments and processed in ImageJ using the PZFociEZ macro. All image analysis parameters were kept constant for each pair of mock and irradiated sample. As with previous studies, cells positive for RAD51 and phospho-H2AX foci are defined as having ≥5 foci per nucleus.[44,71] HR competent cells had greater than two-fold increase in percentage of RAD51-foci positive cells.[65,70] More than 100 cells from at least three fields were analyzed for each sample. All irradiated samples exhibited greater than two-fold increase in the percentage of phospho-H2AX foci positive cells.

**SNP array analysis**

Chromosomal alterations (deletion, amplification, uniparental disomy) involving candidate genes in a cohort of primary myeloproliferative neoplasm cases were interrogated from results previously described.[103] The list of genes in recurrently disrupted chromosome regions was analyzed using the gene ontology (GO) EASE algorithm to identify affected biological pathways.[105]

**Methylation-specific PCR (MSP), quantitative MSP (qMSP)**

Genomic DNA was bisulfite-treated with the EZ DNA Methylation Kit (Zymo Research). The initial MSP screen was performed as previously described.[106] qMSP for *BRCA1* promoter methylation, normalized to beta-actin levels, was carried out using the iTaq SYBR Green mix and 300nM of each primer. qMSP, MSP primer sequences and annealing temperatures are listed in supplemental Table 1. Cycling conditions are $95^0$C for 5min, followed by 40 cycles of $95^0$C for 5sec and $64^0$C for 60sec. Melt curve readings were recorded at $0.5^0$C increments from $65^0$C to $95^0$C. The controls for unmethylated and methylated templates were bisulfite-treated samples from normal lymphocyte DNA and CpG Methylated Jurkat genomic DNA (NEB) respectively. Methylated samples are defined as amplicons with melting temperatures matching that of the methylated control.

**Quantitative real-time PCR (qRT-PCR)**

cDNA was synthesized from 1µg RNA isolated from samples processed within 6h after collection using the iScript Reverse Transcription Supermix (Bio-Rad). Transcript levels were measured in triplicate with the CFX96 Touch Real-Time PCR Detection System in a 15µl reaction including 1µl diluted cDNA, 250nM of each primer and 7.5µl SsoAdvanced SYBR Green mix (Bio-Rad). Primer sequences are listed in Table S1. *BRCA1* expression was quantified using the $2^{-\Delta\Delta Ct}$ method after normalizing to *GAPDH*.

**microRNA expression analysis**

200ng of total RNA was reverse-transcribed using the qScript™ microRNA cDNA Synthesis Kit (Quanta BioSciences) and real-time quantitative analysis was performed in 20µl reaction volume containing SsoAdvanced SYBR Green mix (Bio-Rad), 10µM PerfeCTa Universal PCR primer and 10µM PerfeCTa miRNA assay primer in the CFX96 Touch Real-Time PCR Detection System according to manufacturer's instructions. *miR-155* levels were normalized to *RNU6B* and quantified using the $2^{-\Delta\Delta Ct}$ method.

**Cell culture and reagents**

OCI-AML3 were grown in RPMI media supplemented with 20% FBS, 100units/ml pencillin and 100µg/ml streptomycin. HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS. All cells were maintained at $37^0$C, 5% $CO_2$ in humidified atmosphere. For HDACi treatment, cells were treated with suberoylanilide hydroxamic acid (SAHA) for 72h.

**Lentivirus generation and transduction**

HEK293 cells were transfected with BRCA1-specific or non-targeting shRNA (Sigma), psPAX2 packaging and pMD2.G envelope DNA. The culture medium was replaced after 12h, and supernatant containing lentivirus was collected at 24h and 48h. Concentrated lentivirus stock and 8µg/ml polybrene were added to OCI-AML3 and HL-60 cells and spun at 2400rpm for 60min. The supernatant was discarded and the cell pellet was re-suspended in culture media. After 48h, transduced cells were selected with 2µg/ml puromycin for seven days and repression confirmed by qRT-PCR.

**Cell viability assay for PARP inhibitor sensitivity**

2500 cells were seeded in triplicate in a 96-well plate and treated with PARP inhibitor ABT-888 (ApexBio) at 0.01% final DMSO concentration for 72h. Cell viability was determined using the Metabolic Substrate RealTime-Glo™ MT assay (Promega).

## Results

### Identification of Alterations in HR Genes

Inactivation of tumor suppressor genes in cancer often involves genetic alterations, loss of chromosome regions and abnormal promoter hypermethylation. In myeloid malignancies, mutations in HR genes are infrequent and have not shown a conclusive association with functional disruption of HR.[2,67] Using EASE to identify biological pathways enriched for genomic loss, we next reexamined SNP-A results from a previous study of 144 MPN patients, but found no association for the homologous recombination pathway of statistical significance ($p$=0.121).[93] Since hematological disorders display frequent alterations in CpG methylation,[107-110] we explored whether epigenetic alterations could produce HR defects. *BRCA1* CpG island methylation has been found to be predictive of sensitivity to PARP inhibitors in breast cancer,[111] and another study demonstrated a high incidence of this epigenetic change in treatment related AML.[112] As a first approach to determine which HR genes might be altered by promoter region methylation, we examined previously collected MPN, AML and acute lymphoid leukemia (ALL) samples.[102,103]. As reported in supplemental Table 2, we observed CpG island promoter methylation of *BRCA1* (MPN: 6/66, 9.09%; AML: 2/26, 7.69%), *FANC-C* (AML: 1/30, 3.33%) and *FANC-L* (AML: 1/30, 3.33%). These epigenetic events were absent in ALL samples and normal lymphocytes, suggesting a specific association with myeloid malignant cells. The remaining HR genes (*BRCA2*, *ATM*, *ATR*, and *FANC-A*) showed no evidence of promoter CpG island methylation (supplemental Table 2).

**Homologous Recombination is Frequently Defective in Myeloid Neoplasms**

In response to DNA DSBs, the formation of RAD51 foci at sites of damage has been well established as a marker of HR repair.[65,70-72] However, previous studies of the HR pathway in myeloid malignancies required extended treatment (24h) with DNA damaging agents, which limits the investigation to a small number of transformed cases due to the stress of prolonged *ex vivo* culture.[67,68] In addition, the influence of drug uptake and metabolism cannot be controlled. We have previously characterized impaired RAD51 foci induction in a small cohort of MPN samples that was associated with PARP inhibitor sensitivity.[93] Here, we expanded our study to include the pre-leukemic conditions (MDS, MPN, MDS/MPNs) for a more comprehensive investigation of HR defects in myeloid neoplasms (supplemental Table 3). To minimize the effects associated with lengthy drug incubations, we exposed primary cells to ionizing radiation, which has been previously shown to induce similar levels of DSBs to DNA damaging drugs.[104] We also restricted our assessment to fresh viable samples processed within 6h after collection. We performed this assay in 21 patient samples (14 MDS/MPNs, 5 MPNs, 1 sAML, 1 therapy-related MDS). For each pair of mock and radiated samples, we performed immunofluorescence and confocal microscopy for γH2AX and RAD51 foci as markers of DSB and HR respectively (Figure 1A, supplemental Figure S1). The percentage of both γH2AX- and RAD51-foci positive cells (≥5 foci per cell) was determined in mock and radiated fractions (Figure 1B top). In order to account for differences in baseline characteristics, we determined the fold change after radiation (Figure 1B bottom). All irradiated samples demonstrated a greater than two-fold increase in percentage of γH2AX-foci positive cells post-radiation, confirming that irradiation generated

reproducible and robust DNA DSB damage. A two-fold induction of RAD51-foci positive cells post-radiation was used to define functional HR. The failure to achieve a two-fold induction of RAD51-positve cells has previously been demonstrated to correlate with PARP inhibitor sensitivity.[65,70] Using this improved approach that reduces subjectivity and variation during sample and image processing, we observed diminished RAD51 foci induction in 9 of 21 (43%) samples, demonstrating a high prevalence of impaired HR in myeloid malignancies (Figure 1C).

**Epigenetic Silencing of *BRCA1* is Associated with HR Defects**

To determine if promoter region hypermethylation of these genes functionally produced the HR defective phenotype, we examined 96 DNA samples from 51 unique patients and carried out a focused methylation analysis of *BRCA1*, *FANC-C*, *FANC-L* since our pilot study had suggested these genes could be affected by promoter region methylation (Figure 2). Sequential samples for some patients allowed us to monitor molecular changes over time. There was no promoter methylation of *FANC-C* and *FANC-L* in any of these patients, suggesting that epigenetic silencing of these genes is rare in myeloid neoplasms (data not shown), and could not account for the observed frequency of HR defects. We refined the methylation detection for *BRCA1* to exclude amplification of unconverted DNA, using a real-time quantitative MSP (qMSP) assay with melt curve analysis to compare the melting temperature of each amplicon to that of the *in vitro* methylated positive control. With this approach, as shown in Figure 2A, we detected *BRCA1* methylation in 22.9% (22/96) of the samples, corresponding to 12 of 51 (23.5%) patients. Among the patients with sequential samples for analysis, we found promoter

methylation to be unchanged over time in most patients, with persistent absence of methylation in 14 patients and retained methylation in four cases (10, 12, 28, 33). There was loss of *BRCA1* methylation in subsequent samples for two patients (19, 23), while in two cases (1, 17) we observed *BRCA1* methylation in samples collected at later time points (Figure 2A). A review of the patient history revealed that patient 23 had received 5'azacytidine, a demethylating agent, after the first sample was collected, potentially explaining that loss of methylation. The onset of BRCA methylation seen in patients 1 and 17 suggests that acquiring BRCA1 methylation may be a feature of disease progression.

To determine whether promoter methylation of *BRCA1* was associated with transcriptional repression, we examined *BRCA1* expression in 88 samples with RNA available. We found statistically significant lower expression in cases with *BRCA1* promoter hypermethylation compared to those samples lacking methylation ($P<0.05$, unpaired *t*-test) (Figure 2B). In the subset of samples with HR status, we found a statistically significant association between *BRCA1* methylation and HR defects ($P<0.05$, Fisher's Exact Test, Table 2). We next classified *BRCA1* expression with HR and *BRCA1* promoter methylation according to HR and promoter methylation status (Figure 2C). HR defective samples with *BRCA1* CpG island methylation exhibited significantly lower *BRCA1* gene expression compared to HR competent cases with unmethylated BRCA1 ($P<0.05$, unpaired *t*-test). Of note, all four *BRCA1* methylated samples were defective for HR, suggesting that this epigenetic silencing event disrupted HR repair (Figure 2C). For HR defective samples that were unmethylated for *BRCA1*, we observed varying levels of

*BRCA1* expression suggestive of dysregulation in other HR genes and the possibility of *BRCA1* repression through alternative mechanisms (mutations, chromatin repression) as the cause of HR defect. We also observed in patient 17, the acquisition of hypermethylation of *BRCA1* promoter at the later time-point was associated with disruption of HR repair, while the initial sample from this patient was unmethylated and had intact HR repair (supplemental Figure 2). Overall, promoter methylation and silencing of *BRCA1* was strongly associated with HR defects, showing for the first time how this epigenetic event disrupts the HR pathway in myeloid malignancy.

**Repression of *BRCA1* Expression Results in PARPi Sensitivity**

To determine if HR defects due to *BRCA1* inactivation can be exploited by therapies that selectively target HR deficient cancer cells, we examined *in vitro* sensitivity to the PARP inhibitor ABT-888 (veliparib). *BRCA1* inactivation has been associated with increased sensitivity to PARP inhibition in breast and ovarian cancer due to the synthetic lethal killing of HR defective cancer cells.[43] To determine whether *BRCA1* loss would create a similar sensitive phenotype in myeloid cells, we stably expressed two independent short hairpin RNAs targeting *BRCA1* in the OCI-AML3 cell line that is unmethylated for *BRCA1* promoter and was previously characterized as HR competent.[67] This resulted in *BRCA1* repression and impaired RAD51 foci induction, recapitulating the association observed in primary samples (Figure 3A, 3B). We then measured cell viability after 72h exposure to ABT-888 and observed a statistically significant decrease in $IC_{50}$ values with *BRCA1* knockdown (scrambled control: 16.2μM; *BRCA1* sh34: 7.5μM; *BRCA1* sh49: 5.72μM) ($P<0.0001$, F-test), indicative of increased sensitivity to PARP inhibition

(Figure 3C). These results demonstrate that loss of *BRCA1* impairs HR and leads to increased sensitivity to PARP inhibition, pointing to a therapeutic approach for the subset of myeloid neoplasm patients with HR defects and *BRCA1* promoter methylation as an epigenetic biomarker to identify potential responders to this therapy.

### *BRCA1* Represses *miR-155* Expression via HDACs

In addition to this important role for *BRCA1* in HR, we hypothesized that there may be additional consequences of *BRCA1* inactivation in myeloid neoplastic cells that confer transformed cells a growth advantage. It has been shown in breast cancer that BRCA1 directly represses miR-155 by recruiting a repressive complex that includes histone deacetylase 2 (HDAC2) to the *miR-155* promoter.[113] This is of relevance in myeloid malignancies since previous studies have identified miR-155 as an oncomiR that is frequently over-expressed and promotes myeloid lineage expansion of hematopoietic stem cells.[98,99] However, an association between *miR-155* up-regulation and *BRCA1* loss in myeloid malignancies has not been reported. Therefore, we examined *miR-155* expression in patient samples and found a statistically significant inverse correlation with *BRCA1* expression (Spearman correlation coefficient $r$:-0.245, $P$<0.05) (Figure 4A), suggesting that BRCA1 loss was associated with increased *miR-155* expression. To directly test whether this was caused by *BRCA1* loss, we examined *miR-155* expression following repression of *BRCA1* in OCI-AML3 cells and observed a >1.5-fold increase in miR-*155* levels (Figure 4B). Since the mechanism proposed by which *BRCA1* represses *miR-155* involves HDAC2, we treated OCI-AML3 cells with SAHA, a pan-HDAC inhibitor. This resulted in a dose-dependent increase *in miR-155* expression following

SAHA treatment, further implicating BRCA1 in repression of miR-155 as mediated via HDACs (Figure 4C).

**Loss of *miR-155* repression is associated with reduction in myeloid differentiation genes**

As an oncogenic microRNA, multiple miR-155 targets have been proposed, including *PU.1* and *SHIP1*.[97,100,114,115] These proteins have well-established roles in myeloid differentiation and both have been reported to be frequently downregulated in myeloid malignancies.[100,101,116] Therefore, we examined if increased *miR-155* observed in *BRCA1* silenced myeloid malignancies was associated with expression of these target genes. We indeed found a strong inverse correlation of both PU.1 and *SHIP1* transcript levels (Figure 4D, 4E) with expression of *miR-155* (*PU.1*: Spearman correlation $r$:-0.264, $P<0.05$; *SHIP1*: Spearman correlation $r$:-0.364, $P<0.005$). This suggests a mechanism involving loss of *BRCA1* repression leading to *miR-155* up-regulation, promoting myeloid transformation through reduction in key mediators of myeloid differentiation.

## Discussion

Myeloid malignancies often present with significant clinical heterogeneity, which presents a challenge for diagnosis and the identification of genetic aberrations that can be effectively targeted.[117] Recently, the favorable activity of PARP inhibitors on tumors with HR deficiencies has generated further interest in characterizing disease systems for these defects. Given that myeloid malignancies are characterized by gross chromosomal instability, PARP inhibitors represent promising therapies for these patients given the synthetic lethal effect in sparing normal tissues.[67,118,119] A detailed investigation of the HR repair pathway within the diverse spectrum of myeloid disease is needed. In this study, we investigated acute leukemia and pre-leukemic disorders (MPN, MDS, mixed MDS/MPN) to determine the prevalence of HR functional defects. To address the lack of a short-term, *ex vivo* HR assay for hematological samples, we have developed an ionizing radiation-based assay and determined HR repair function by measuring RAD51 foci recruitment to DSB sites. This modification has allowed us to comprehensively and objectively query HR competency and conclude that a high proportion (43%) of myeloid malignancies exhibit HR defects.

Increasingly, it is acknowledged that HR repair defect can arise in sporadic cancers from molecular aberrations aside from mutations in BRCA1/2.[120] To identify the molecular underpinning of this HR defect, we considered the various mechanisms of disrupting HR genes, including mutation, loss of heterozygosity and promoter methylation. Multiple genetic studies have described germ-line mutations in HR repair genes in hematologic

neoplasms (*ATM*, *BLM*, *Fanconi Anemia*), but these events are infrequent in sporadic myeloid disorders.[2,67,121] Analysis of genomic regions lost in MPN also failed to highlight the HR pathway as significantly disrupted. *BRCA1* promoter methylation was previously identified in a significant proportion of treatment related AML patients that correlated with complex karyotypes, but the HR pathway was not evaluated in that study.[112] Here, we reported *BRCA1* CpG island methylation in a significant proportion of patient samples. More importantly, this is the first study correlating epigenetic silencing of BRCA1 with HR defects in primary samples, strongly highlighting *BRCA1* promoter methylation as a promising biomarker for HR defects and resultant sensitivity to PARP inhibition. In clinical research settings, we acknowledge that it may not be feasible to obtain clinical samples in a timely manner required to perform the HR assay and gene expression analyses. Hence the ability to detect stable epigenetic changes in a simple and sensitive manner favors promoter hypermethylation as a clinical biomarker.

The HR and BRCA1 methylation assays demonstrated here could be developed into valuable companion tools for evaluating hematological samples in a clinical setting to inform decisions for incorporating PARP inhibitors. The recent approval of olaparib for *BRCA* mutated ovarian cancer based on improved progression free survival illustrates the clinical benefit of this agent for tumors that lack HR, a strategy that is actively pursued in multiple malignancies.[96,122,123] The ABT-888 $IC_{50}$ values of *BRCA1*-repressed cells are below the single-dose $C_{max}$ readings recorded in preclinical animal models, highlighting a therapeutic window to apply this agent for this population.[124] Two clinical trials are ongoing at the Johns Hopkins Hospital (ClinicalTrials.gov Identifier: NCT01139970,

NCT00588991) to evaluate the effects of adding PARP inhibitor ABT-888 in combination with chemotherapeutic agents for hematological disorders.

Despite the numerous genes involved in HR, the exclusivity and prevalence of *BRCA1* epigenetic inactivation in myeloid neoplasms suggest the potential for additional roles in which loss of BRCA1 drives disease progression in addition to defective DSB repair. The recent finding that *BRCA1* represses oncogenic *miR-155* provides a possible role, given that *miR-155* is frequently up-regulated in myeloid malignancies and studies showing ectopic overexpression of *miR-155* in mouse models results in a myeloproliferative-like phenotype.[97-99,113,114] Our findings of an inverse correlation of *BRCA1* and *miR-155* transcripts in patient samples, increased *miR-155* levels in the *BRCA1*-silenced cells, and confirmation that this is repression mediated by *HDACs* are in agreement with previous findings in breast cancers suggesting a transcriptional repressor role for BRCA1.[113] Given the prominent role of miR-155 in normal and pathogenic hematological systems, and the direct targeting of PU.1 and SHIP1 by this oncogenic miRNA, our result highlight a HR-independent mechanism by which BRCA1 loss drives myeloid disease progression.

In conclusion, our findings suggest that a subset of myeloid malignancies exhibit HR defects, which can be mediated by epigenetic silencing of *BRCA1*, and are therefore susceptible to the synthetic lethal killing effects of PARP inhibitors. Further investigation is warranted to determine if HR status and *BRCA1* methylation can predict favorable outcomes with PARP inhibition in myeloid malignancies, which may usher in a novel therapy concept for these patients who currently lack effective curative treatment options.

## Figure Legends

**Figure 1. Homologous recombination repair is impaired in myeloid neoplasms.**
(A) Representative confocal images after processing with ImageJ macro PZFociEZ to highlight foci (right). Robust induction of RAD51 foci was observed in HR competent cells (HR+), but not in cells defective for HR (HR-). Images were obtained with a Nikon C1si confocal laser-scanning microscope using a 100X.1.4 N.A. oil-immersion objective. Excitation wavelengths were 408nm (DAPI), 488nm (γH2AX), and 561nm (RAD51). The scale bar represents two microns. (B) Quantification of RAD51-foci positive (≥ 5 foci) cells in samples shown in (A). HR competent cells are defined as having greater than 2-fold increase in percentage of RAD51 positive cells after irradiation, indicated by the red-dotted line. (C) Summary result of HR functional assay for primary patient samples shows defective HR in 9 of 21 (42.9%) samples.

**Figure 2. Epigenetic silencing of BRCA1 is linked to HR repair defects.**
(A) BRCA1 qMSP results. Unique patient samples are labeled numerically. Multiple samples from the same patient are indicated alphabetically, in chronological order. (B) Dot-plot of *BRCA1* expression relative to normal controls (NL) classified by promoter methylation. The line represents the median. *, $P<0.05$ (unpaired *t*-test with Welch's correction) (C) Dot-plot of *BRCA1* expression according to HR repair status and promoter hypermethylation. HR defective, *BRCA1* methylated samples exhibit lower *BRCA1* expression compared to HR competent, *BRCA1* unmethylated samples. UM: unmethylated, M: methylated. The line represents the median. *, $P<0.05$ (unpaired *t*-test)

**Figure 3. Stable Repression of *BRCA1* induces HR defects and increased PARP inhibitor ABT-888 sensitivity.**

(A) *BRCA1* shRNA-mediated repression in OCI-AML3 cells confirmed by qPCR. (B) BRCA1-repressed cells failed to induce RAD51 foci with irradiation. Error bars represent SEM. (C) Increased sensitivity to PARP inhibitor ABT-888 was observed with *BRCA1*-knockdown. Cell viability was accessed 72h after adding ABT-888. The data is shown with SD as error bars.

**Figure 4. *BRCA1* loss relieves HDAC-mediated repression of *miR-155* that results in decreased expression of downstream miR-155 targets implicated in myeloid disease.**

(A) *BRCA1* and *miR-155* expression levels were plotted for primary samples, showing an inverse correlation. (B) *miR-155* expression is elevated in *BRCA1*-silenced OCI-AML3 cells. (C) Treatment with HDAC inhibitor SAHA results in increased *miR-155* expression. Error bars in (A) and (B) represent SEM of three independent experiments. (D) *PU.1* and (E) *SHIP1* expression were plotted against that of *miR-155* after $\log_2$ transformation of the transcript levels relative to normal lymphocytes. The number of pairs (*n*), Spearman correlation coefficient (*r*) and *P* values are shown.

**Supplemental Figure 1. A significant proportion of myeloid neoplasm samples display abnormal HR repair.** Image analysis of foci-positive nuclei was performed for each pair of mock and radiated sample, using identical image processing parameters. Cells positive for phospho-H2AX and

RAD51 foci are defined as having ≥5 foci per nucleus. More than 100 cells from at least three fields were analyzed for each sample. All irradiated samples exhibited greater than two-fold increase in the percentage of phospho-H2AX foci positive cells.

**Supplemental Figure S2. *BRCA1* qMSP results of samples assayed for HR status.** Results are indicated for samples where *BRCA1* qMSP and HR assay were performed.

**Figure 1. Homologous recombination repair is impaired in myeloid neoplasms.**

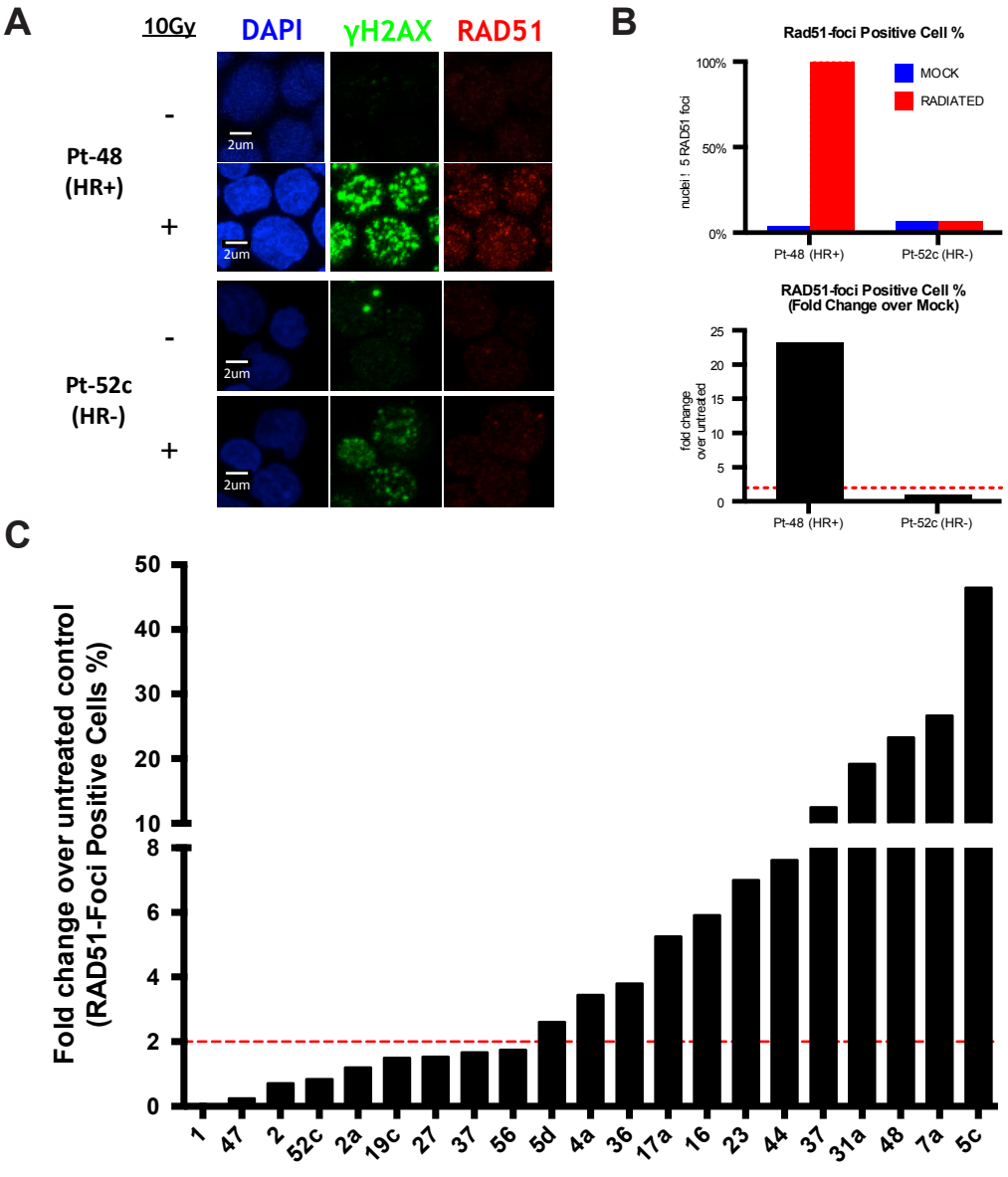


**Figure 2. Epigenetic silencing of *BRCA1* is linked to HR repair defects.**

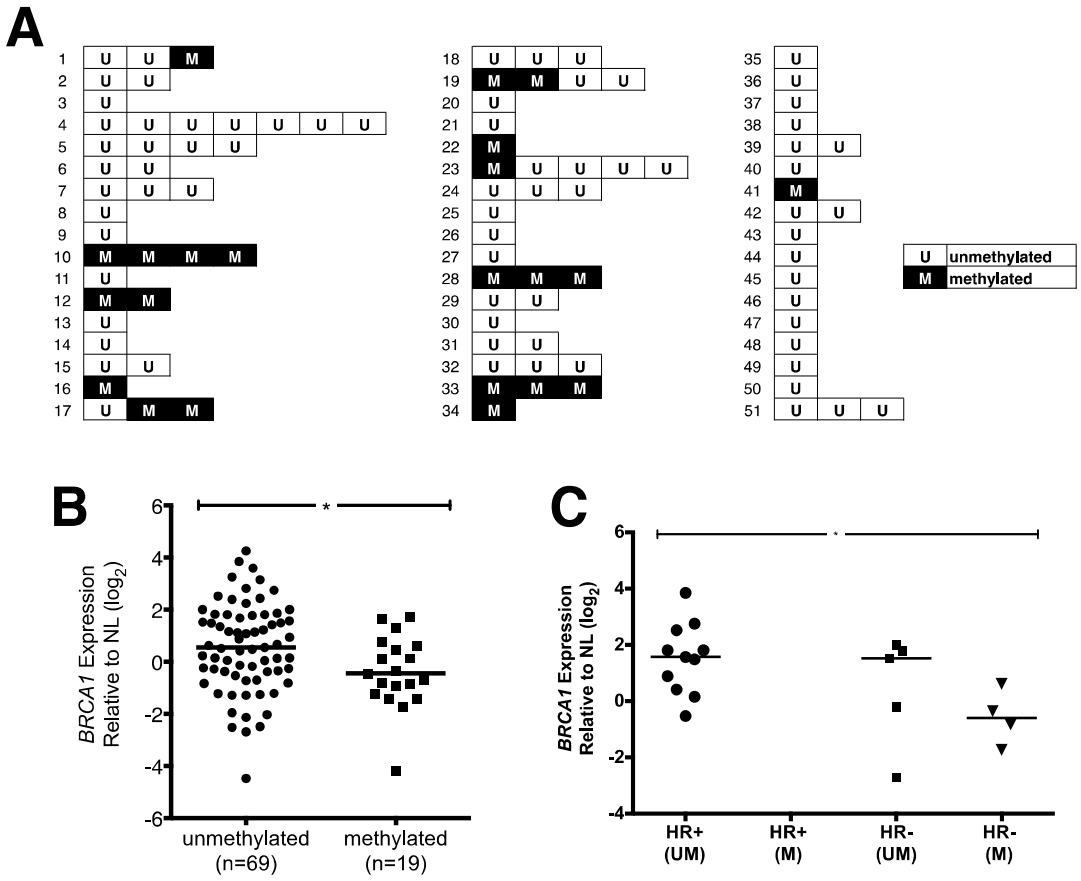


**Figure 3. Stable Repression of *BRCA1* induces HR defects and increased PARP inhibitor ABT-888 sensitivity.**

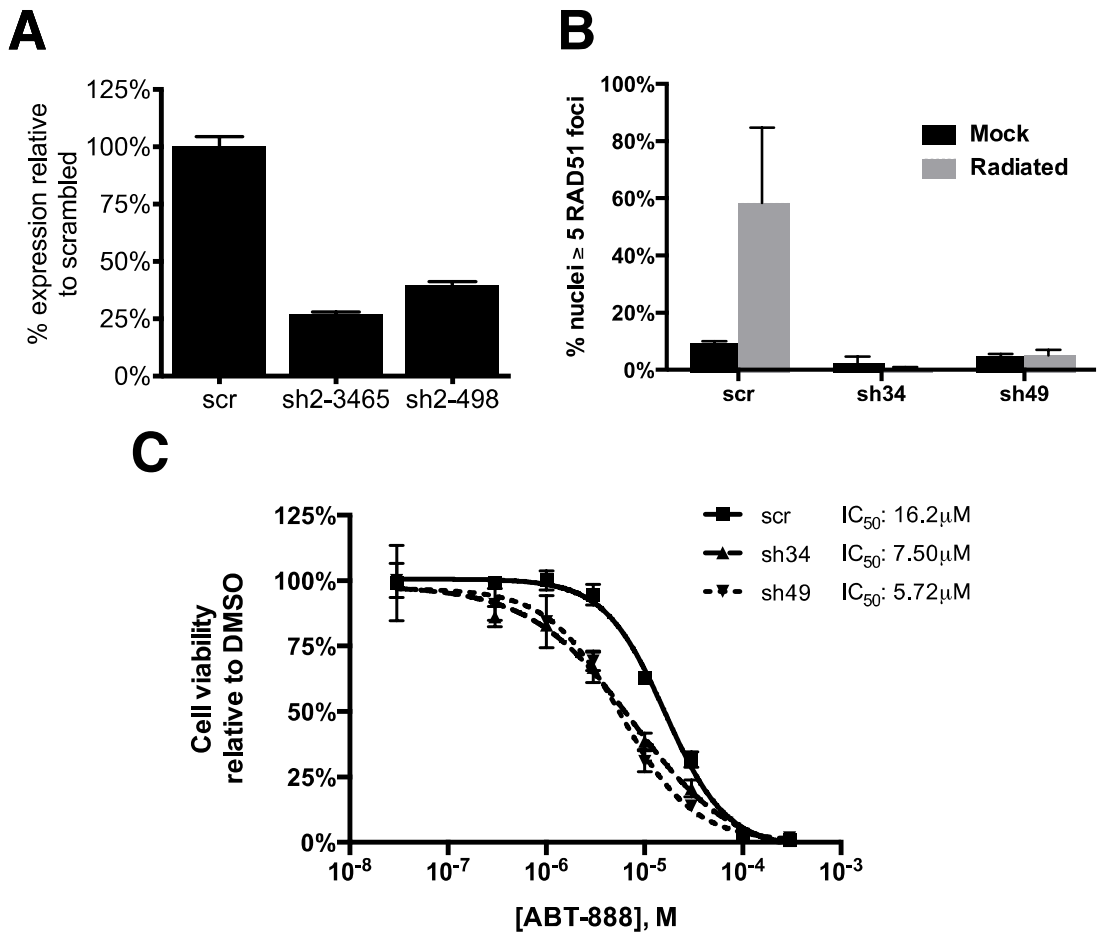
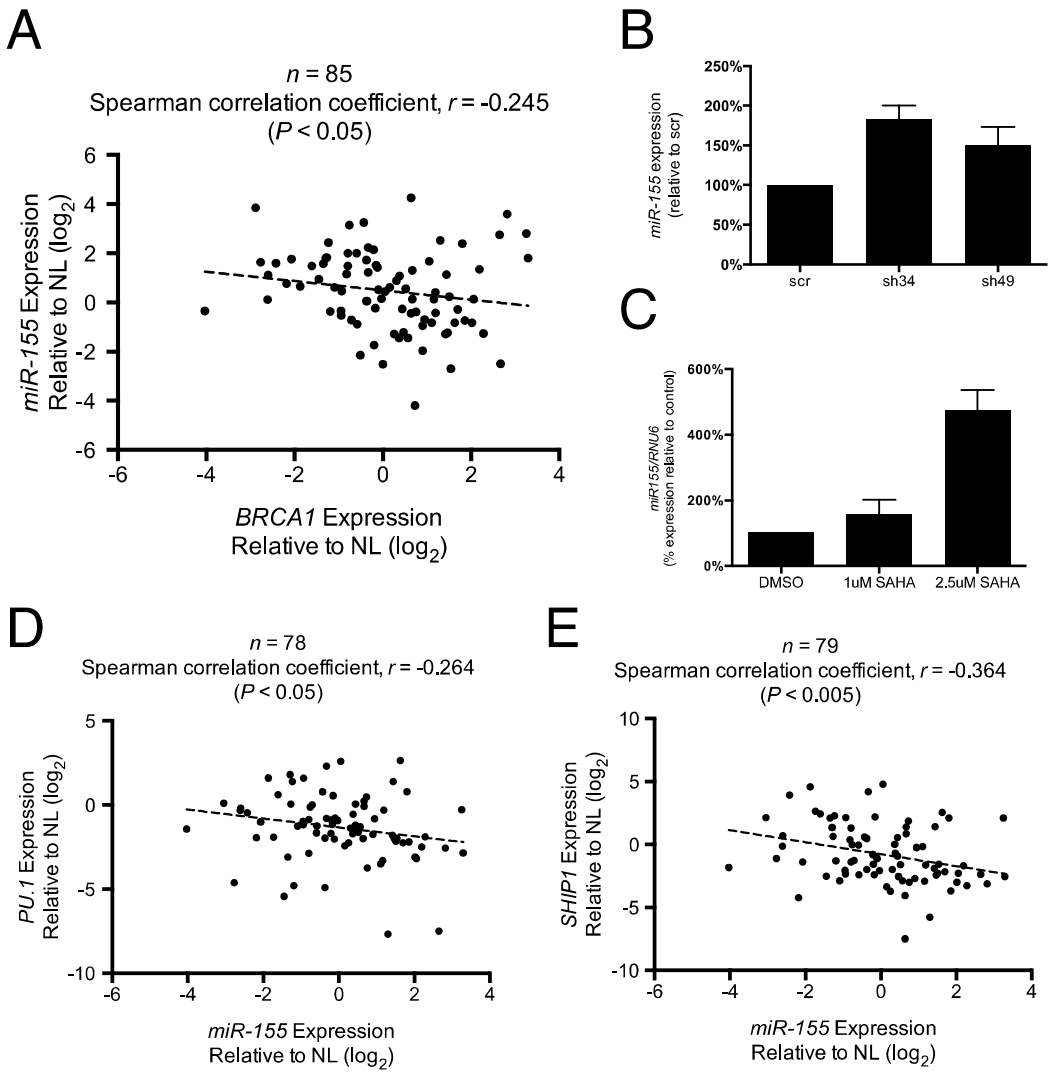
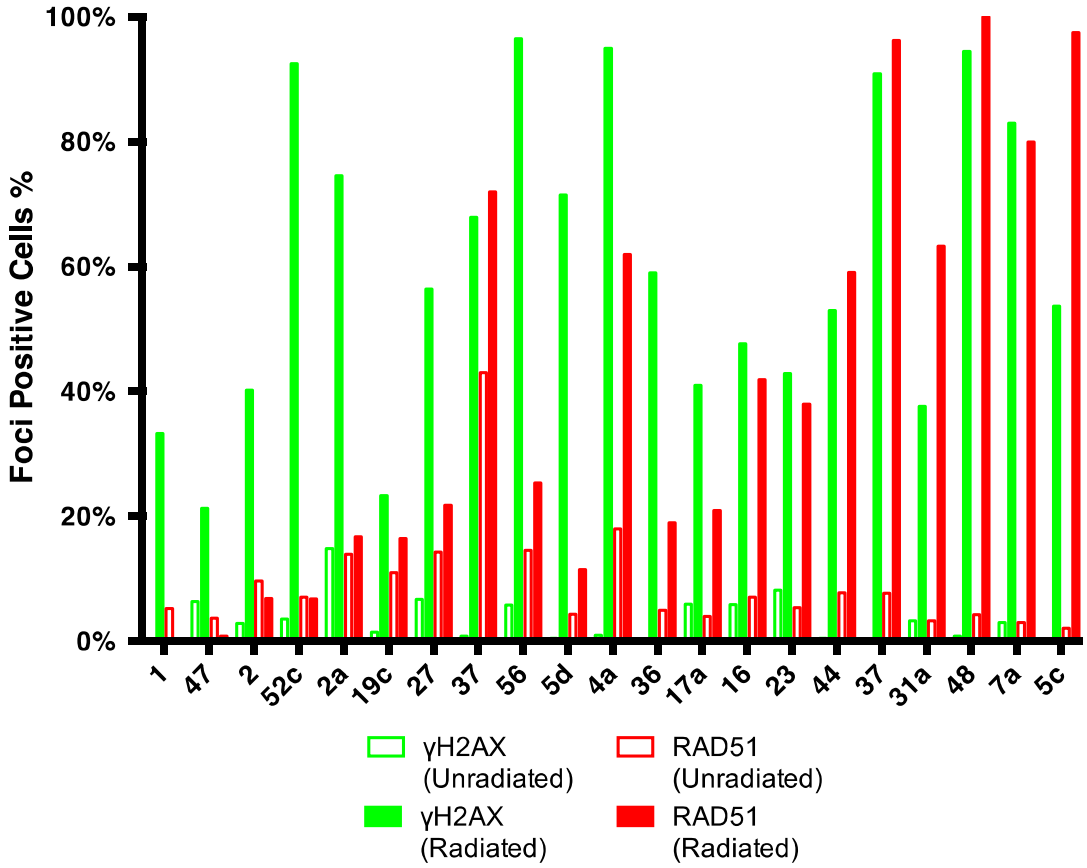


**Figure 4. *BRCA1* loss relieves HDAC-mediated repression of *miR-155* that results in decreased expression of downstream miR-155 targets implicated in myeloid disease.**



**Supplemental Figure 1. A significant proportion of myeloid neoplasm samples display abnormal HR repair.**



**Supplemental Figure S2. *BRCA1* qMSP results of samples assayed for HR status.**

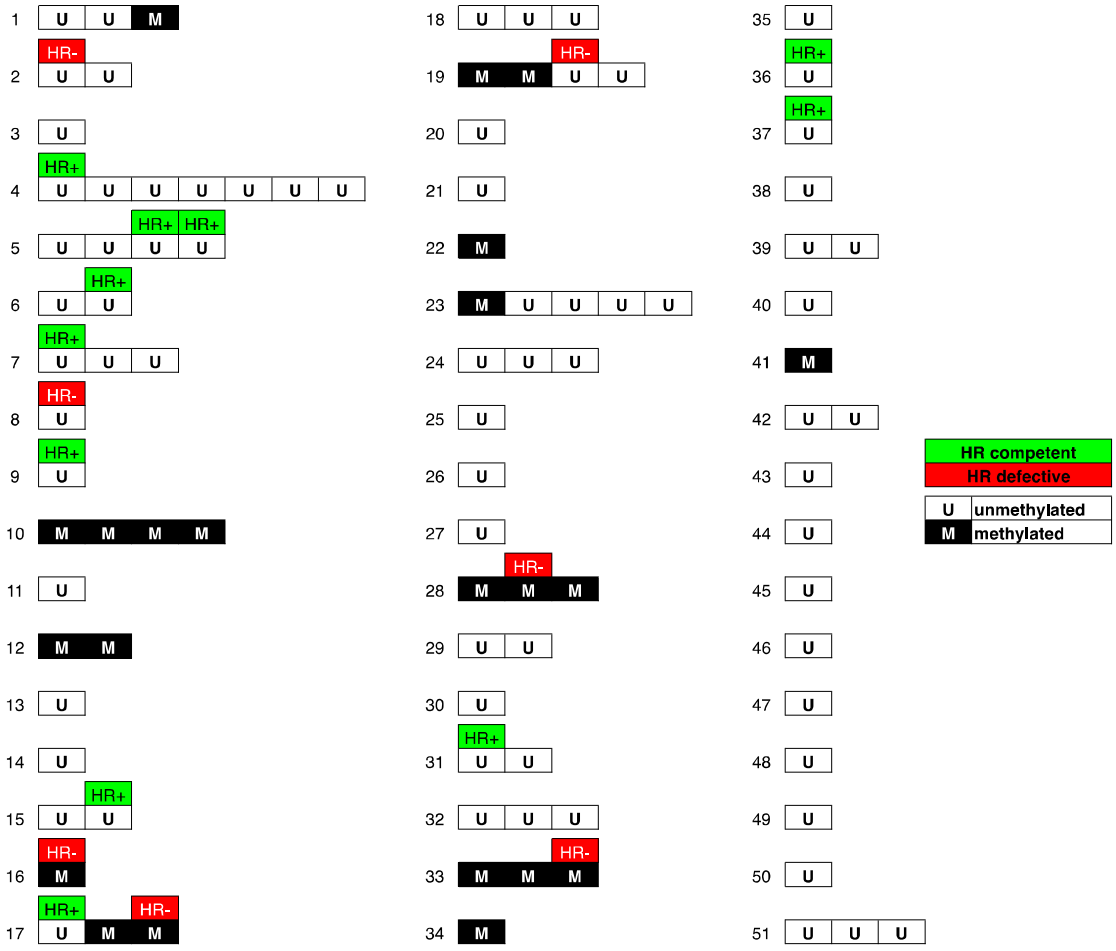


**Table 1. Study cohort characteristics.**

| | MDS/MPN | MDS/AML | MPN |
|---|---|---|---|
| Unique Patients | **32** | **9** | **16** |
| Samples | | | |
| gDNA (methylation) | **74** | **12** | **26** |
| RNA (gene expression) | **60** | **9** | |
| Mononuclear cells (HR) | **17** | | **6** |

**Table 2. Association between *BRCA1* methylation and HR defects.**

| | HR competent | HR defect | |
|---|---|---|---|
| MDS/MPN | **7** | **12** | ***P*<0.05, Fisher's Exact Test** |
| MPN | **3** | **1** | |

## Supplemental Table 1. Primer sequences for qMSP, MSP, RT-qPCR

| qMSP | Primer sequence 5'-3' | Tm |
|---|---|---|
| ***M-BRCA1*** | | 64°C |
| **sense** | GGG TGG TTA ATT TAG AGT TTC GAG AGA CG | |
| **antisense** | AAC GAA CTC ACG CCG CGC AAT CG | |
| ***Actin*** | | 64°C |
| **sense** | TAG GGA GTA TAT AGG TTG GGGAAGTT | |
| **antisense** | AACACACAATAACAAACACAAATTCAC | |
| **MSP** | **Primer sequence 5'-3'** | **Tm** |
| ***FANC-C*** | | 60°C |
| **UM sense** | TGTTGTTGTAATTTTGTTTTTGTTGGGGTTTTTG | |
| **UM antisense** | AACACAACCAAAACTCTAACAAAAATAAACCCA | |
| **M sense** | GACCAAAACTCTAACGAAAATAAACCCG | |
| **M antisense** | TTGTTGTAATTTTGTTTTCGTCGGGGTTTTC | |
| ***FANC-L*** | | 64°C |
| **UM sense** | TTTTATTATTGGTTGGGGAGTTTTGATGTTGTTTTTTGTAGAGTG | |
| **UM antisense** | AACTCTAAACCTACTAAATCCTACACATACACAATCCACTA ACA | |
| **M sense** | TTGGGGAGTTTTGATGTCGTTTTTCGTAGAGC | |
| **M antisense** | CTCTAAACCTACTAAATCCTACACATACGCAATCCGCTAAC G | |
| ***FANC-F*** | | 62°C |
| **UM sense** | TATGTTAGTATTTGGGATTTTGTTATTGTGTGTTG | |
| **UM antisense** | CTAATTATACAACCACCACTCCAAAACCATACA | |
| **M sense** | TATTTGGGATTTCGTTATCGTGCGTC | |
| **M antisense** | CGCCGCTCCAAAACCGTACG | |
| ***BRCA2*** | | 64°C |
| **UM sense** | TGATTTTTGGGTGGTGTGTGTGTTGTG | |
| **UM antisense** | CAAAAACACAACAATACCACAACAACTCCA | |
| **M sense** | TTTTCGGGTGGTGCGTGTGTTGCG | |
| **M antisense** | CGCAACAATACCACAACGACTCCG | |
| ***FANC-N*** | | 62°C |
| **UM sense** | GATTTTGGTTATTTTTGGTTGTTTTTTTATTTTTG | |
| **UM antisense** | AACCATAAACAACCAAACAATAAAACAAAACCA | |
| **M sense** | ATTTCGGTTATTTTCGGTCGTTTTTTTATTTTC | |
| **M antisense** | GACCGAACAATAAAACGAAACCG | |
| ***ATM*** | | |
| **UM sense** | TAGGTGTGTTTGTTTTGATGGGTTG | 62°C |
| **UM antisense** | AACAACCACAAAACACAATATACCCATACA | |
| **M sense** | CGTTTGTTTCGACGGGTC | |
| **M antisense** | CGCAAAACAGATATACCCATACG | |
| ***FANC-A*** | | 62°C |
| **UM sense** | TAGGTGTTTTTTTTAGGATTAATATGGTTATGATG | |
| **UM antisense** | CAAACACACACACCCATTAACTAACAACA | |
| **M sense** | GGCGTTTTTTTTAGGATTAATATGGTTACGAC | |

| | | |
|---|---|---|
| **M antisense** | CACACCCGTTAACTAACGACG | |
| ***BLOOM*** | | 62°C |
| **UM sense** | GAAGTAGTTAATTGGAATAGGTAAGTTTTTGGTG | |
| **UM antisense** | CCTCACACACAAACTCCTAACAAACA | |
| **M sense** | TAGTTAATCGGAATAGGTAAGTTTTCGGC | |
| **M antisense** | CACGCAAACTCCTAACGAACG | |
| ***FANC-D2*** | | 64°C |
| **UM sense** | GTGAGTTTTTTTTTATTGGGGTGTAGTTGTTTTTTTTTGATGTTG | |
| **UM antisense** | CTCACCACTACAACAAAATTCTATACTAACCAAACATAAAAACA | |
| **M sense** | GAGTTTTTTTTTATCGGGGCGTAGTTGTTTTTTTTTGACGTC | |
| **M antisense** | TCACCGCTACAACAAAATTCTATACTAACCGAACATAAAAACG | |
| ***FANC-E*** | | 62°C |
| **UM sense** | TTGGTTTTATGTTTAGGTTTTGTTTTTGGTTGTG | |
| **UM antisense** | CTATTACAAACTCTCCTCAACATTAAAAAACCA | |
| **M sense** | TTTAGGTTTCGTTTTCGGTCGC | |
| **M antisense** | TCTCCTCGACGTTAAAAAACCG | |
| ***FANC-G*** | | 62°C |
| **UM sense** | TTTGTTTATTTTTTTTTGAGGTTGTGGTTTTTGTG | |
| **UM antisense** | CAAACAAACTTAAACAAACTAAAAACCAAAATCA | |
| **M sense** | GTTTATTTTTTTTCGAGGTTGTGGTTTTCGC | |
| **M antisense** | AAACAAACTTAAACGAACTAAAAACCGAAATCG | |
| ***FANC-I*** | | 62°C |
| **UM sense** | TGTTTTTTTTTGATTGTGAGTTGGGATGTTTTTG | |
| **UM antisense** | ACTTCCACCATCCAAATACCTACAAACA | |
| **M sense** | TTTTTTTTCGATTGTGAGTTGGGACGTTTTC | |
| **M antisense** | CCGTCCGAATACCTACGAACG | |
| ***FANC-J*** | | 62°C |
| **UM sense** | GGATTTGTTGGAGTGGTGATAATTTTTTGTTGTG | |
| **UM antisense** | AATCCCAATACAAAATAAAAAAACAAAAACTTCA | |
| **M sense** | AGTGGCGATAATTTTTCGTCGC | |
| **M antisense** | CCGATACGAAATAAAAAAACGAAAACTTCG | |
| ***ATR*** | | 58°C |
| **UM sense** | GTTTGGTGTTGAGAAAGAAGTTTGATGTGATTTG | |
| **UM antisense** | TTCTACATAAAACCCAAAAAAAAACCAAAAAACCA | |
| **M sense** | TGTTGAGAAAGAAGTTTGACGCGATTC | |
| **M antisense** | TACATAAAACCCGAAAAAAACCGAAAAACCG | |

| **RT-qPCR** | **Primer sequence 5'-3'** | **Tm** |
|---|---|---|
| ***BRCA1*** | | 60°C |
| **sense** | CAACATGCCCACAGATCAAC | |
| ***antisense*** | ATGGAAGCCATTGTCCTCTG | |
| **GAPDH** | | 60°C |
| **sense** | GAAGGTCGGAGTCAACGGATTT | |
| ***antisense*** | ATGGGTGGAATCATATTGGAAC | |

## Chapter 3: Conclusion

### Targeting HR Defects in Myeloid Malignancies

The recent approval of the PARP inhibitor olaparib for advanced ovarian cancers now presents a promising therapeutic option for other malignancies with HR defects.[122] Hence, there is a concerted effort to uncover predictive biomarkers of defective HR to better stratify patients for targeted therapeutics instead of the conventional cancer-type specific approach. However, in contrast to breast and ovarian cancers, detailed studies of HR repair in myeloid malignancies are wanting in part due to the lack of optimal HR assays designed for primary myeloid malignant samples. This needs to be addressed urgently as there is a significant unmet clinical need for effective therapies with reduced toxicities compared to current regimes. Given the frequent chromosomal aberrations reported for theses diseases, there is an suggestions that defective HR could be a common mechanism for genomic alterations across multiple myeloid disorders. [27,28] Hence, we set out to develop a short-term, *ex vivo* assay by modifying existing HR assays to determine the presence and prevalence of defects in HR, using ionizing radiation to rapidly induce DSBs and quantifying RAD51 foci induction indicative of proficient HR. Given the limitations of previous assays because of the variability associated with imaging analysis, we performed these measurements with consistent parameters between each pair of mock and radiated sample using an ImageJ macro. By implementing

these adaptations, we believe this assay can be more widely implemented in research settings to provide a more comprehensive and accurate understanding of HR repair. With this technique, we were able to assay 21 primary samples and found frequent incidence of impaired RAD51 foci induction (9/21 samples, 43%). These findings are promising for investigators choosing to focus on targeting HR repair deficiency in this group of diseases. Since there are few molecular targeted agents available for such patients, PARP inhibitors represent another possible therapeutic approach to halt disease progression in MPN, MDS and MDS/MPN patients who are at high risk of acute leukemic transformation.[2] Previous studies have alluded to the potential of incorporating PARP inhibitors into AML therapy, but these were often restricted to a small number of patient samples that insufficiently answer the prevalence of HR defects in myeloid malignancies.[67,68] To our knowledge, this is the first study looking at defective HR across multiple myeloid neoplasms that suggests this deficiency is not limited to transformed AML cases.

However, we also recognize obstacles to translate this technique into routine clinical assays: 1) the requirement for sufficient number of viable cells (at least $2x10^6$), and 2) the lack of a cell cycle specific marker to identify dividing cells. For myeloid conditions such as MDS and MF where it might not be feasible to obtain sufficient patient tissue, adjustments will be needed to scale down this approach so that it may be used with a limited

number of cells. During the course of this work, a study was published that incorporated geminin staining as a marker of proliferating cells.[72] This may allow normalization for cells of differing rates of cell growth. Nonetheless, given the ease of access to fresh viable malignant cells in most myeloid neoplasms compared to solid cancers, HR assays could be extremely informative in the context of clinical trials with PARP inhibitors.

At the same time, we investigated the molecular mechanism underlying HR defects, focusing on epigenetic silencing of HR genes given the low incidence of mutations in this group of diseases.[2,67,121] We observed promoter DNA methylation of *BRCA1* in 23% (22/96) of the patient samples that is closely linked to defective HR. This result not only provides a mechanistic explanation for disrupted HR repair, but also offers a robust biomarker that makes easier the process of identifying patients who are potentially amenable to PARPi therapies. More importantly, these patients with *BRCA1* promoter methylation and HR defects may not be considered eligible for trials involving PARP inhibitors based on the current criteria of *BRCA* mutations or clinical features that are associated with *BRCA* mutations.[47] Our results here support the addition of DNA methylation of *BRCA1* for patient selection, which has been shown to exhibit similar gene expression profiles as *BRCA1* mutated cancers, to extend the presumed benefits of PARP inhibitors to a larger patient population.[125] The observation of HR defective samples in our study that do not exhibit *BRCA1* promoter methylation suggests alterations in other HR genes or alternate

forms (chromatin repression) of *BRCA1* repression. Going forward, it will be informative to perform whole-genome analysis (sequencing, DNA methylation, SNP-A, ChIP-seq) to unravel these alternative mechanisms.

### *BRCA1* Loss and *miR-155* Up-regulation in Myeloid Neoplasms

In our study, we also observed an inverse correlation between *BRCA1* and *miR-155* levels in both patient samples and the *BRCA1*-silenced OCI-AML3 cells. This provides critical insight into the regulation of miR-155, which is highly expressed in myeloid malignancies and elevated miR-155 have been associated with inferior outcomes in AML patients.[97,100,114,115] This provides an additional mechanism other than defects in HR by which *BRCA1* loss contributes to myeloid transformation, and such loss could lead to cells that are more aggressive in nature. Retrospective analyses of patient DNA with clinical outcomes will help shed light on this finding.

### Acquired Resistance

Since PARP inhibitors may exhibit reduced major side effects by selectively targeting HR defective cancers, these agents are suitable for long-term treatments to convert lethal cancers into chronic diseases. However, it is acknowledged that tumors can develop resistance to PARP inhibitors over time through multiple mechanisms: 1) increased drug efflux or 2) restoration of HR repair by reversion of BRCA1 activity. One solution is to inhibit P-glycoprotein activity that is known to increase drug efflux. For resistant tumors with partially restored HR, a possible approach is using drug

combinations such as platinum drugs, although this would induce more severe side effects that may not be tolerable in elderly myeloid neoplasm patients.[126]

**Future Directions**

Collectively, this body of work highlights myeloid neoplasms as a potential disease model amenable to PARP inhibitors, with the appropriate functional assay or DNA methylation analysis. This approach is currently being evaluated in clinical trials incorporating PARP inhibitors in myeloid malignancies (J0783, PI: Keith Pratz, MD; J1051, PI: Ivana Gojo, MD). Further studies are required to correlate clinical response to functional status of HR repair and DNA methylation of *BRCA1*.

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126. Bouwman P, Jonkers J. Molecular pathways: how can BRCA-mutated tumors become resistant to PARP inhibitors? *Clin Cancer Res*. 2014;20(3):540-547.

**Curriculum Vitae**

# Weijie Poh

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117 Park Drive, Apt D. Boston, MA 02215.

- Early-stage, translational research scientist in DNA repair and epigenetic biomarkers.
- Highly collaborative researcher across clinical and bench research, cumulating in seven co-authored peer-reviewed publications and four manuscripts in preparation.
- Designed and processed more than 150 samples from myeloid neoplasm patients.
- Developed *ex vivo* confocal imaging assay for DNA repair in primary samples.
- Established protocol for examining epigenetic silencing of DNA repair genes in patient samples.
- Supported clinical trials evaluating poly (ADP) ribose polymerase (PARP) inhibitors in myeloid malignancies.
- Oversaw marketing and research operations in Philadelphia-based biotech startup (Vector Biolabs, Inc).

## Academic and Research Experience

**2010-June 2015** Johns Hopkins University. School of Medicine. Pathobiology Ph.D. Program.

- Conferred Margaret Lee Fellowship for outstanding Singaporean applicants.

**Mentor:** James G. Herman, M.D.

**Thesis:** *Epigenetic Silencing of BRCA1 Creates Homologous Recombination Repair Defects and Impaired Differentiation through Elevated miR-155 Expression in Myeloid Neoplasms*

- Developed clinical collaboration and primary tissue processing protocol with the Johns Hopkins Hospital clinicians.
- Discovered functional DNA repair defect in myeloid neoplasm that is attributed to epigenetic silencing of HR genes.
- Demonstrated that DNA repair defects and epigenetic silencing predicts favorable response to PARP inhibitors.
- Attended weekly clinical conferences discussing lymphoma and myeloid malignancy patient cases.

**Significance:** Epigenetic biomarkers can be used to select for responders to PARP inhibitors from myeloid neoplasm patients who

currently lack curative therapy. This approach is currently explored in clinical trials at the Johns Hopkins Hospital.

**Translational Rotation:** Johns Hopkins Hospital. Surgical Pathology. Jonathan Epstein, MD.

- Observed surgical sample processing from gross examination to frozen/fixed tissue sectioning and pathologist evaluation.

**Translational Rotation:** Johns Hopkins Hospital. Division of Hematology. Ivana Gojo, MD.

- Shadowed hematologist interactions with elderly leukemia patients in managing and monitoring disease symptoms.

**Academic and Research Experience (continued)**

**2009-2010** Agency for Science, Technology and Research (A*STAR), Singapore. Institute of Medical Biology. Singapore Oncogenome Project. Research Officer.

- Designed projects to examine effect of tyrosine kinase inhibitors in hepatocellular carcinoma cell lines.
- Optimized assays for fluorescence-activated cell sorting of cancer stem cells.

**2008-2009:** Agency for Science, Technology and Research (A*STAR), Singapore. Institute of Medical Biology. Singapore Oncogenome Project. Honors Year Research Student.
**Mentors**: Axel Ullrich, Ph.D., Han Kiat Ho, Ph.D.
**Thesis**: *Klotho-beta overexpression as a novel target for suppressing proliferation and fibroblast growth factor receptor-4 signaling in hepatocellular carcinoma*

- Analyzed gene expression in paired normal and tumor tissues from hepatocellular carcinoma patients.
- Determined a liver tissue specific co-receptor protein in the FGFR pathway is upregulated in HCC samples.
- Demonstrated increased expression of CD133 and CD44, markers associated with cancer stem cells, with prolonged treatment of FGFR4 kinase inhibitor.

**Significance**: This work highlights a tissue-restricted protein in the FGFR pathway that can be targeted by chemotherapy to minimize systemic toxicities.

**2007-2008:** Agency for Science, Technology and Research (A*STAR). Institute of Molecular and Cell Biology. Molecular Virology and Viral Pathogenesis Lab, Undergraduate Research Student.
**Mentor:** Ding Xiang Liu, Ph.D.
**Thesis:** *Identification of RIG-I caspase activation recruitment domain (CARD) Interacting Cellular Proteins*

- Generated mutant RIG-I deficient in IRF3 activation by mutagenesis PCR cloning.
- Identified novel interacting partners to RNA sensor protein RIG-I by conducting protein immunoprecipitation followed by mass spectrometry.

**Significance:** Documented a novel interaction of HSP70 with RIG-I with possible role in ubiquitin-mediated degradation.

## Teaching Experience

***Graduate Student Instructor. Pathology for Graduate Students: Basic Mechanisms. 2011***
Led lectures on cancer pathobiology to Johns Hopkins Pathobiology graduate students.

## Technical Skills

- Isolating primary mononuclear cells from bone marrow aspirates and peripheral blood.
- Developing an *ex vivo* radiation assay in primary leukocytes to access homologous recombination.
- Optimizing immunofluorescence staining protocol to detect irradiation induced foci.
- Designing methylation-specific PCR (MSP) to detect promoter methylation of DNA repair gene.
- PCR-cloning based bisulfite sequencing.
- Reverse transcriptase quantitative PCR (RT-qPCR) detection of gene and microRNA expression.
- Cell culture of adherent and suspension cell lines.
- Generating shRNA-expressing lentivirus for transduction into acute leukemic cell (AML) lines.
- Plasmid cloning and bacterial transformation to generate fusion gene constructs.

- Real time cell viability assays to monitor drug sensitivity to drug treatment with gene repression.
- Treatment with histone deacetylase inhibitors to monitor changes in gene expression and histone acetylation.
- Chromatin immunoprecipitation (ChIP) qPCR for histone marks.

## External Poster Presentations

***American Society of Hematology Annual Meeting. 2014.***
**W. Poh**, A. R. Moliterno, K. W. Pratz, I. Gojo, M. A. McDevitt and J. G. Herman "Epigenetic Silencing of *BRCA1* Is Linked to Homologous Recombination Repair Defects and Elevated *mir-155* Expression in Myeloid Neoplasms."

***American Association of Cancer Research Annual Meeting. 2013.***
**W. Poh**, R. L. Dilley, M. A. Healey, A. R. Moliterno, K. W. Pratz, M. A. McDevitt and J. G. Herman. "*BRCA1* promoter methylation and homologous recombination repair status in primary chronic myeloid neoplasms."

## Awards and Honors

***First Place: Local Medical Product (JHU Entrepreneurship Week). 2014.***
Medical and Education Perspectives (MEP), BME EDGE, Innovation Factory.
Headed PathoVax, based on a broadly protective Human Papillomavirus (HPV) vaccine, that beat over 20 teams to win the Local Medical Product category. Awarded $1000 cash and $5000 for commercialization.

***Student Travel Award. 2014.***
Graduate Student Association, Johns Hopkins University.
Awarded $200 for poster presentation at the American Society of Hematology Meeting 2014.

***AAAS/Science Program for Excellence in Science Student Fellowship. 2011.***
Awarded two-year membership in AAAS.

***Margaret Lee Fellowship. 2010.***
Department of Pathology, Johns Hopkins University
One of three students selected from Singapore and Korea to receive funding for graduate education at the Johns Hopkins Pathobiology Ph.D. program.

**Publications**

**W. Poh**, A. R. Moliterno, K. W. Pratz, M. A. McDevitt, J. G. Herman. "Epigenetic silencing of *BRCA1* is linked to homologous recombination repair defects and elevated *mir-155* expression in myeloid neoplasms." (Manuscript in Preparation)

T. R. Pisanic II, P. Athamanolap, **W. Poh**, J. G. Herman, T. Wang. "Quasi-Digital high-resolution melt for ultrasensitive detection and analysis of rare epiallelic variants." (Manuscript in preparation)

C. K. Lee, **W. Poh**, M. A. Healey, E. S. Schafer, P. Brown, J. G. Herman. "Pcft Is Silenced By DNA Methylation In Pediatric Acute Lymphoblastic Leukemia Resulting In Decreased Methotrexate Uptake." (Manuscript in preparation)

M. A. Healey, **W. Poh**, V. Winnepennenickx, C. Hooker, J. G. Herman. "Methylation of MMP11 is an independent negative prognostic marker for survival in melanoma." (Manuscript in preparation)

R. L. Dilley, **W. Poh**, D. E. Gladstone, J. G. Herman, M. M. Showel, J. E. Karp, M. A. McDevitt and K. W. Pratz (2014). "Poly (ADP-ribose) polymerase inhibitor CEP-8983 synergizes with bendamustine in chronic lymphocytic leukemia cells *in vitro*." Leukemia Research 38(3): 411-417.

Y. Jiao, R. Yonescu, G. J. Offerhaus, D. S. Klimstra, A. Maitra, J. R. Eshleman, J. G. Herman, **W. Poh**, L. Pelosof, C. L. Wolfgang, B. Vogelstein, K. W. Kinzler, R. H. Hruban, N. Papadopoulos and L. D. Wood (2014). "Whole-exome sequencing of pancreatic neoplasms with acinar differentiation." The Journal of Pathology 232(4): 428-435.

S. N. Shah, L. Cope, **W. Poh**, A. Belton, S. Roy, C. C. Talbot, Jr., S. Sukumar, D. L. Huso and L. M. Resar (2013). "HMGA1: a master regulator of tumor progression in triple-negative breast cancer cells." PLoS One 8(5): e63419.

C. M. Heaphy, K. C. Schreck, E. Raabe, X. G. Mao, P. An, Q. Chu, **W. Poh**, Y. Jiao, F. J. Rodriguez, Y. Odia, A. K. Meeker and C. G. Eberhart (2013). "A glioblastoma neurosphere line with alternative lengthening of telomeres." Acta Neuropathologica 126(4): 607-608.

**W. Poh**, W. Wong, H. Ong, M. O. Aung, S. G. Lim, B. T. Chua and H. K. Ho (2012). "Klotho-beta overexpression as a novel target for suppressing proliferation and fibroblast growth factor receptor-4 signaling in hepatocellular carcinoma." Molecular Cancer 11: 14.

A. Schuldenfrei, A. Belton, J. Kowalski, C. C. Talbot, Jr., F. Di Cello, **W. Poh**, H. L. Tsai, S. N. Shah, T. H. Huso, D. L. Huso and L. M. Resar (2011). "HMGA1 drives stem cell, inflammatory pathway, and cell cycle progression genes during lymphoid

tumorigenesis." BMC Genomics 12: 549.

B. T. Chua, S. J. Lim, S. C. Tham, **W. J. Poh** and A. Ullrich (2010). "Somatic mutation in the ACK1 ubiquitin association domain enhances oncogenic signaling through EGFR regulation in renal cancer derived cells." Molecular Oncology 4(4): 323-334.

## Professional Activities

***Finalist, Johns Hopkins Business and Consulting Club Mini-Case Competition. 2014.***
Johns Hopkins Business and Consulting Club (JHBCC)
Part of two-man team selected as finalist (out of 16 teams). Given three hours to analyze and present profitability recommendations for Tesla.

***The Columbia Graduate Consulting Club 2014 Case Competition. 2014.***
The Columbia Graduate Consulting Club, Columbia University, New York
Part of five-person team selected for finals at the Columbia University. Analyzed business case involving mobile payment technology and presented recommendations to industry consultants within four hours.

***3rd Annual Spring Biotech Business Case Competition. 2013.***
Johns Hopkins Business and Consulting Club (JHBCC)
Part of three-person team analyzing case of medical device OEM exploring expansion into novel product categories.