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Award Number: W81XWH-06-1-0323

TITLE: Checkpoint Functions of the BRCA1/BARD1 Tumor Suppressor

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New York, NY 10032

REPORT DATE: July 2008

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-07-2008	Annual Summary	1 Jul 2007 – 30 Jun 2008

4. TITLE AND SUBTITLE

Checkpoint Functions of the BRCA1/BARD1 Tumor Suppressor

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0323

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Columbia University
New York, NY 10032

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT No abstract provided.

15. SUBJECT TERMS
BARD1; BRCA1; siRNA; DNA damage, checkpoints

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
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INTRODUCTION

The objective of my research is to study the role of BRCA1 in breast cancer by determining how BARD1 phosphorylation affects the checkpoint and DNA repair functions of the BRCA1/BARD1 heterodimer [1].

In my original application, I proposed to examine the role of BARD1 phosphorylation in the checkpoint functions of BRCA1 by generating and characterizing isogenic subclones of HCT116 cells that express different knock-in alleles of BARD1. Subsequently, however, I also tested the feasibility of an alternative approach based on siRNA-mediated depletion of endogenous BARD1 coupled to transient reconstitution with exogenous BARD1. This approach has several advantages over the original knock-in strategy. First, since it involves transient transfection of a cell population, this approach is not susceptible to artifacts that arise due to clonal variation. Second, unlike the knock-in strategy, which is restricted to certain pseudo-diploid cell lines such as HCT116, this approach can be applied to a broad range of cell types. Third, this approach is more facile since it does not require the laborious process of generating stable knock-in subclones by targeted gene recombination.

To evaluate the role of BARD1 phosphorylation in homology-directed repair (HDR) of double-strand DNA breaks (DSB), Bard1-null mouse mammary tumor cells bearing an HDR reporter construct (DR-GFP) were transfected with expression vectors encoding either wildtype or mutant forms of human BARD1. Briefly, this reporter contains two distinct nonfunctional copies of the GFP gene: one copy (*Sce*GFP) is disrupted by the recognition site for the rare-cutting endonuclease I-*Sce*I, while the other copy (*i*GFP) encodes only an internal region of GFP. However, a functional GFP gene can be regenerated when a DSB break triggered by I-*Sce*I cleavage of the SceGFP is repaired by HDR utilizing *i*GFP as a template, and such events can be quantified by flow cytometry. Using this assay, we previously showed that transfection of these Bard1-null cells with an expression vector encoding human BARD1 induces an approximately 5-fold increase in HDR function [2]. This approach allowed me to determine if BARD1 phosphorylation mutations impair BARD1 function in HDR.

BODY

The checkpoint functions of BRCA1

To implement the siRNA-mediated approach to examine checkpoint function, I first designed two distinct siRNAs (siRNAs A and B) that can greatly reduce endogenous BARD1 expression (>90%) in a variety of cell lines (Figure 1). Second, by site-directed mutagenesis I introduced non-coding mutations into our BARD1 mammalian expression plasmids that render the resultant mRNAs resistant to knockdown by either siRNA A or B. With these reagents, we should be able to test whether BARD1 phosphorylation is required for specific

checkpoint functions of BRCA1. For example, a BRCA1-dependent function, such as the IR-induced G_2 accumulation checkpoint, should be ablated by siRNA-mediated BARD1 knockdown, either as a direct consequence of BARD1 inactivation or as an indirect consequence of BRCA1 instability in the absence of BARD1. In either case, transfection of the siRNA-treated cells with a siRNA-resistant vector encoding wildtype BARD1 should rescue the checkpoint. If, however, a specific phosphorylation site (for example, S251) is required for the G_2 accumulation checkpoint, then transfection with a siRNA-resistant vector encoding S251A-mutant BARD1 should restore the expression levels of BRCA1 but not rescue checkpoint activity. Thus, by reconstituting siRNA-treated cells with siRNA-resistant expression vectors encoding the full panel of wildtype and phosphorylation site mutant BARD1 polypeptides, we should be able to identify the precise requirements for BARD1 phosphorylation in checkpoint function. A similar strategy was used successfully by Yu *et al.* to demonstrate a requirement for BACH1 phosphorylation in the same IR-induced G_2 accumulation checkpoint [3]. Moreover, this strategy was used not only to study the G_2 accumulation checkpoint (Task 1), but also a variety of other checkpoints including the IR-induced transient G_2/M, spindle assembly, and mitotic exit checkpoints.

The IR-induced G_2 accumulation checkpoint: In evaluating the effect of BARD1 knockdown on the G_2 accumulation checkpoint in 293 cells, siRNA-mediated knockdown of BRCA1 was included as a positive control, since BRCA1 is known to be required for this checkpoint [3]. Approximately 48 hrs and 72 hrs post-second transfection, one set of cells was irradiated with 10 Gy, while a second set was mock treated. After three hours at 37^{o}C, both treated and mock-treated cells were incubated for 15 hours with nocodazole (1ug/mL) to arrest cells in mitosis. The cells were then fixed with 70% ethanol and placed at -20^{o}C overnight. The mitotic population of each culture was then measured by flow cytometric analysis after staining with propidium iodide and the mitotic marker, phospho-histone H3. As expected, knockdown of BRCA1 caused a defect in activation of the G_2 accumulation checkpoint. Significantly, BARD1 knockdown also induced a checkpoint defect, as illustrated by an ~5-10 fold increase in the percentage of cells that entered mitosis following IR treatment relative to control cells (Figure 2).

To confirm that the observed checkpoint defect is due to BARD1 knockdown, and not due to non-specific off-target effects of the siRNAs, we introduced silent mutations into the siRNA-specific targeting regions of a BARD1 mammalian expression vector to render its mRNA product resistant to either the BARD1-specific siRNA A or B. Two mutations, especially if placed together near the middle of the siRNA sequence, are generally sufficient to ablate siRNA-mediated knockdown, although more mutations can only help [4]. In our design of siRNA-resistant BARD1 expression vectors, we were able to introduce 3 or 4 tandem nucleotide changes that disrupted siRNA complementarity but did not alter the coding potential of the vector. Of note, the BARD1 polypeptides encoded by these vectors contain an N-terminal tag of three tandem FLAG epitopes that

allows the endogenous and exogenous (i.e., vector-encoded) forms of BARD1 to be distinguished in rescue experiments. To ascertain whether the G_2 accumulation checkpoint of the BARD1 siRNA-treated cells is due to BARD1 depletion, 293 cells that had been BARD1-depleted by two successive siRNA transfections (with siRNAs A or B) were transiently co-transfected with the appropriate siRNA-resistant BARD1 expression vector. Western blot analysis with a FLAG-specific antibody confirmed successful expression of exogenous BARD1 in siRNA-treated cells (Figure 3). Significantly, these cells displayed an ~ 3-fold decrease in the percentage of mitotic cells after IR exposure, indicating that reconstitution of BARD1-depleted cells with siRNA-resistant wildtype BARD1 provides a partial rescue of the G_2 accumulation checkpoint (Figure 4). Reconstituting BARD1-depleted cells with siRNA-resistant BARD1 polypeptides bearing specific mitotic phosphorylation mutations resulted in an intact G_2 accumulation checkpoint, suggesting that mitotic phosphorylation of BARD1 is not required for this checkpoint (Figure 4).

The results described here were reported as abstracts at the Annual Meeting of the American Association of Cancer Research in April, 2007 [5] and the Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope 2008 Meeting in June, 2008 [6].

The IR-induced transient G_2/M checkpoint: In previous studies, siRNA-mediated depletion in Hela cells has been used successfully to implicate the CtIP and BRCA1 proteins in this cell cycle checkpoint [7]. In our studies, two rounds of siRNA transfections were performed approximately 24 hrs apart and led to efficient knockdown of BARD1 (Figure 5). One set of cells was irradiated with 5 Gy, while a second set was mock treated. After one hour at 37^{o}C, the cells were fixed with 70% ethanol and placed at -20^{o}C overnight. The mitotic population of each culture was then measured by flow cytometric analysis after staining with propidium iodide and the mitotic marker, phospho-histone H3. As expected, knockdown of CtIP caused a defect in activation of the transient G_2/M checkpoint [6]. Significantly, BARD1 knockdown also induced a checkpoint defect, as illustrated by ~2-4 fold increase in the percentage of cells that entered mitosis following IR treatment relative to control cells (Figure 6). Several rescue attempts by simple transient co-transfections of siRNA-resistant cDNA were not effective in this cell line and resulted in apparent toxicity; thus, a lentiviral approach (Invitrogen; as described by Yu et al 2003 [3]) is currently underway to produce stable BARD1 siRNA-resistant cell lines under Blasticidin selection. Once rescue of the IR-induced transient G_2/M checkpoint is completed with stable siRNA-resistant clones, we will test stable lines bearing phospho-mutant forms of BARD1 to determine the role of BARD1 mitotic phosphorylation in this checkpoint.

Other BRCA1-dependent checkpoints: With these systems for depletion and reconstitution of BARD1 expression in place, we will also evaluate the role of BARD1 phosphorylation in two addition checkpoints that are dependent on

BRCA1: The spindle assembly checkpoint and the IR-induced mitotic exit checkpoint.

The DNA repair functions of BRCA1

Previous studies have established that both BRCA1 and BARD1 are required for homology-directed repair (HDR) of double-strand DNA breaks (DSB) [8,9,2]. To evaluate the role of BARD1 phosphorylation in this process, we used a system in which Bard1-null mouse mammary tumor cells (#2-218 cell line) bearing an HDR reporter construct (DR-GFP) were transfected with expression vectors encoding either wildtype or mutant forms of human BARD1 [2]. This reporter contains two distinct nonfunctional copies of the GFP gene: one copy (*Sce*GFP) is disrupted by the recognition site for the rare-cutting endonuclease I-*Sce*I, while the other copy (*i*GFP) encodes only an internal region of GFP. However, a functional GFP gene can be regenerated when a DSB break triggered by I-*Sce*I cleavage of the SceGFP is repaired by HDR utilizing *i*GFP as a template, and such events can be quantified by flow cytometry [10]. Using this assay, we previously showed that transfection of these Bard1-null cells with an expression vector encoding human BARD1 induces an approximately 5-fold increase in HDR function [2]. For our purposes, we generated Fl3-tagged mutants in which the serine residues of the five non-cdk sites (S184, S186, S251, S391, T394) were replaced with alanines (pX5A) to create a phospho-deficient mutant (Figure 7). We also generated a phospho-mimicking mutant in which the same five residues were replaced with aspartic acid (pX5D). Additionally, mutants in which all seven mitotic sites (S148, S184, S186, S251, T299, S391, T394) were mutated to either alanines (pX7A) or aspartic acids (pX7D) were also evaluated (Figure 7). A plasmid bearing the deletion of the BRCT domain in BARD1 (dBRCT) served as a control as this construct is known to be defective in rescuing the HDR defect of these cells [2]. Our studies show that full-length wild-type BARD1 rescues the HDR defect in Bard1-null cells to a comparable extent as any of the phospho-mutants tested (Figure 8). Therefore, although BARD1 itself is required for HDR of DSBs, mitotic phosphorylation of BARD1 appears to be dispensible for this function.

The HDR results described here were reported as part of an abstract at the Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope 2008 Meeting in June, 2008 [6].

KEY RESEARCH ACCOMPLISHMENTS

- Two different BARD1-specific siRNAs (A and B) were designed to have a GC content of 30-50% and a general sequence of AA(19N)TT targeting the coding region of BARD1 mRNA. The chemically synthesized RNA duplexes were also created to have 3' overhanging UU dinucleotides, since these are reported to be most effective in knocking down the intended target [3]. The target sequence of siRNA A lies in coding exons

2/3 of BARD1, while that of siRNA B resides in coding exon 9. siRNA-mediated knockdowns were conducted by transient transfection of 293 and Hela cells with HiPerfect (Qiagen), a reagent that allows for highly effective gene silencing at low siRNA concentrations that minimize off-target effects. In pilot experiments, we observed that two sequential siRNA transfections (8 nM), approximately 24 hours apart, yields a highly effective knockdown (>90%) of BARD1 with either the A or B siRNAs, as compared to cells treated with the non-targeting (negative control) siRNA. The knockdown efficiencies were monitored at 48 hrs post-second transfection by Western analysis in both 293 and Hela cell lines (Figure 1,5).

- BARD1 knockdown in 293 cells results in an IR-induced G_2 accumulation checkpoint defect leading to ~5-10 fold increase in the percentage of cells that enter mitosis following IR damage relative to control cells (Figure 2).
- Reconstitution of BARD1-depleted cells with siRNA-resistant BARD1 polypeptides bearing mutations of specific mitotic phosphorylation sites does not lead to an IR-induced G_2 accumulation checkpoint defect (Figure 4).
- BARD1 depletion in Hela cells leads to a ~2-4 fold increase in the percentage of cells that enter mitosis post-IR treatment in the transient G_2/M checkpoint. (Figure 6).
- Phospho-mutant and phospho-mimicking forms of BARD1 both rescue the HDR defect comparably to the wild-type form of BARD1 in BARD1-null cells indicating that mitotic phosphorylation of BARD1 does not function in this form of DNA repair (Figure 12).

REPORTABLE OUTCOMES

Modi, A.P., A.D. Choudhury, and R. Baer (2007). *Functional Analysis of Mitotic Phosphorylations in BARD1.* American Association for Cancer Research (AACR) Annual Meeting (April 2007; Los Angeles, CA).

Modi, A.P. and R. Baer (2008). *Functional Analysis of Mitotic Phosphorylations in BARD1.* Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope 2008 Meeting (June 2008; Baltimore, MD).

Laufer, M., S.V. Nandula, **A.P. Modi**, S.Wang, M.Jasin, V. V. V. S. Murty, T.Ludwig, and R. Baer (2007). *Structural Requirements for the BARD1 Tumor Suppressor in Chromosomal Stability and Homology-directed DNA Repair.* J. Biol. Chem. 282: 34325-34333.

CONCLUSION

Using siRNA-mediated knockdown, we conclude that BARD1 is required for both the IR-induced G_2 accumulation and transient G_2/M checkpoints. However, phosphorylation of BARD1 does not impact the G_2 accumulation checkpoint. Lentiviral rescue experiments are currently underway to assess the role of BARD1 phosphorylation in the IR-induced transient G_2/M checkpoint, as well as the spindle assembly and IR-induced mitotic exit checkpoints. In addition, although BRCA1 and BARD1 are both required for homology-directed repair of double-strand DNA breaks, phosphorylation of BARD1 appears to be dispensable for this process.

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