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Genetic shRNA screens are nowerful tools for identifying loss of function phenotypes in mammalian cells and can be used to complement						
sequencing efforts in the identification of new drug targets. To perform such screens, we have developed bar coded shDNA libraries in						
retroviral and lentiviral vectors that target the entire human genome. Our proposal aims to use this povel screening technology to identify						
new theraneutic targets for breast cancers harboring RRCA1 mutations. Towards this end, we aim to identify and characterize genes that are						
selectively required for proliferation and survival of breast cancer cells with BRCA1 loss-of-function mutations but not required for viability of						
isogenic breast cancer cells expressing wild-type, functional BRCA1. These genes, called BSI s for BRCA1 Synthetic Lethal genes, are						
essential in cells lac	king BRCA1, and as s	such are prime targets	for therapeutic interv	vention to treat	breast cancers that harbor a loss of	
BRCA1 function. We have accomplished the isolation of isogenic cell lines that differ only in their BRCA1 genes, one with wild-type levels of						
functional BRCA1 ar	nd one with a mutant	BRCA1 gene. We hav	e shown that these c	ells are differen	tially sensitive to DNA damage in the	
form of ionizing radia	ation. Cells carrying a	a mutant BRCA1 are r	nore IR-sensitive that	n their complem	ented wild-type counterparts. We have	
begun to perform the synthetic lethal shRNA screen on these two cell liens using our barcoded shRNA library. This strategy should uncover						
many previously unrecognized targets for therapeutic intervention and has the potential to provide additional. more effective treatment						
options for BRCA1 mutant breast cancer patients.						
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Introduction

Women carrying mutations in the *BRCA1* gene, one of two major breast cancer susceptibility genes, have an up to 80% risk of developing breast cancer in their lifetimes¹. These tumors tend to be highly aggressive medullary carcinomas that lack expression of the estrogen or progesterone hormone receptors and amplification of the *HER-2* oncogene^{2, 3}. As the most successful current breast cancer treatments target either hormone receptors or *HER-2*, *BRCA1* mutant tumors generally have a poor prognosis compared to other breast cancer subtypes⁴.

The BRCA1 protein is a central component of the cellular pathways involved in the response to DNA damage and control of cell cycle checkpoints⁵⁻⁷. BRCA1 contains two well-defined domains – an N-terminal RING domain, involved in ubiquitylating other proteins, and tandem C-terminal BRCT domains, which mediate interaction with phosphorylated proteins⁸⁻¹⁰. BRCA1 can form three mutually exclusive complexes based on BRCT-mediated interactions with Abraxas (ABRA1), BRIP1 (BACH1) and CTIP¹¹⁻¹³. These BRCA1 interacting proteins recruit additional factors that facilitate the diverse functions of these complexes. The BRCA1-Abraxas complex (BRCA1A) also contains RAP80, BRCC36, BRCC45, and NBA1 (MERIT40)^{11, 14-18}. It is recruited to sites of DNA breaks through RAP80 binding to ubiquitylated proteins formed at breaks by the ubiquitin ligases RNF8 and RNF168¹⁹⁻²¹. The BRCA1A complex regulates the G2-M cell cycle checkpoint by regulating the phosphorylation status of the checkpoint kinase Chk1²². The BRCA1B complex, composed of BRCA1, BRIP1 and TOPBP1, regulates both initiation of DNA replication and activation of the replication stress checkpoint²³⁻²⁵. The BRCA1-CTIP complex (BRCA1C) is required for efficient resection of DNA ends to allow loading of the single stranded DNA binding complex RPA and thus repair through homologous recombination^{26, 27}. The numerous functions of BRCA1 complexes highlight the importance of this protein in normal cellular function, and the ways that mutations that interrupt these complexes could lead to tumor formation.

Although *BRCA1* was cloned over 15 years ago, therapeutics that specifically target *BRCA1* mutant cells have not yet been developed. As we discussed in our proposal for this award, we aim to exploit differences between cancer and normal cells to identify new targets for therapeutics that will specifically kill the cancer cells. Tumors can be dependent upon oncogenes harboring gain of function mutations for their survival, which is termed oncogene addiction²⁸. Cancer cells must also cope with increased cellular stresses, such as oxidative stress, DNA damage and aneuploidy, which require an increased dependence on normal cellular stress response pathways. We have termed this dependence on non-oncogenic targets non-oncogene addiction^{29, 30}. Both oncogene and non-oncogene addiction pathways are likely to yield therapeutic targets that can be exploited in the treatment of *BRCA1* mutant tumors.

In this award, we proposed using a genetic loss-of-function screen to identify genes that are essential to *BRCA1* mutant cell proliferation and survival. This screen is based on viral shRNA libraries developed in our lab, in collaboration with the lab of Greg Hannon, that target the entire human genome with an average of three shRNAs per gene^{31, 32}.

These libraries can be introduced into cells in a pooled format, allowing for rapid screening of the effects of knock-down of each gene in the genome. In this study, we are introducing the genomic shRNA library into a breast cancer cell line that harbors a homozygous mutation in *BRCA1* that leads to truncation of the protein and loss of a C-terminal BRCT repeat, as well as an isogenic cell line that has been reconstituted for expression of wild-type *BRCA1*. In this manner, we aim to identify and characterize genes that are selectively required for the proliferation and survival of cells expressing mutant BRCA1 protein. These genes, which we call BSLs (for BRCA1 Synthetic Lethal genes), will be prime targets for therapeutics to treat *BRCA1* mutant breast cancers.

Body

Generation of a cloned isogenic pair of breast cancer cell lines

The HCC1937 breast cancer cell line harbors a homozygous mutation in *BRCA1*, 5382insC, which results in truncation of the BRCA1 protein and loss of one of the BRCT repeats³³. These cells are hypersensitive to ionizing radiation and display a defect in repair of DNA double strand breaks^{34, 35}. Complementation of these cells with wild-type BRCA1 decreases IR sensitivity and restores normal DNA repair kinetics^{34, 35}. In the original proposal, we planned to generate newly complemented cells with a neomycinselectable wild-type BRCA1 construct, to enable us to use our puromycin-selectable version of the shRNA library. However, difficulty in generating these cells led us to obtain HCC1937 cells from Ralph Scully that were already complemented with BRCA1 and have been previously characterized³⁴. These cells express a bicistronic retroviral construct that contains both wild-type BRCA1 and green fluorescent protein (GFP), which can be used as a marker of retroviral expression. We also obtained HCC1937 cells expression.



Figure 1. Expression of reconstituted wild-type BRCA1. Expression of BRCA1 was analyzed in the parental bulk HCC1937-GFP and HCC1937-BRCA1 populations, as well as in several subclones from each population. Two BRCA1 antibodies, MS110 and C20, were used. GFP expression was also assayed. The clones GFP #1 and BRCA1 #1 were selected for use in the screen.

The BRCA1 complemented cells were a polyclonal population that had been selected by fluorescence-activated cell sorting. To ensure that each cell we were infecting with the shRNA library was expressing the same level of BRCA1, we subcloned the cells by limiting dilution to obtain a clonal population of reconstituted cells. We also subcloned the GFP control cells and selected clones with growth kinetics similar to the BRCA1reconstituted cells. These clones were screened by immunoblotting for expression of both BRCA1 and GFP (Figure 1). The blots were probed with two BRCA1 antibodies, both of which showed a low level of endogenous BRCA1 present in the parental HCC1937 cells and an increased level of BRCA1 in the reconstituted cells. All of the cells expressed similar levels of GFP. A reconstituted clone with high BRCA1 expression was selected for use in the screen, along with a GFP-only expressing clone. To determine whether the BRCA1 reconstitution was functional, we assayed its ability to decrease the IR sensitivity of the HCC1937 cell line. To accomplish this, we performed a colony formation assay, comparing the number of colonies formed on plates that received no treatment to plates that received three gray of gamma irradiation. We found that, indeed, the expression of wild-type BRCA1 in our clonal population was able to rescue the radiation sensitivity of HCC1937 (Figure 2).



HCC1951BRCA

Figure 2. Colony survival is increased in clonal population expressing wild-type BRCA1. HCC1937-BRCA1 and HCC1937-GFP clones were plated at 1000 cells/plate and then treated with 3 Gy gamma irradiation or left untreated. Colonies were counted two weeks after irradiation.

Genome-wide shRNA screening for BRCA1 synthetic lethal genes

HCC1931-EFP

We are currently performing genome-wide shRNA lethality screens using our isogenic clonal populations of BRCA1-mutant (HCC1937-GFP) and BRCA1-wild-type reconstituted (HCC1937-BRCA1) cells (Figure 3). Our library is divided into six pools of approximately 13,000 shRNAs each. Each pool is introduced into the cell populations in triplicate, at a representation of 1000 cells per shRNA and a multiplicity of infection (MOI) of 1. This ensures that each cell is infected with approximately one shRNA and that each shRNA is introduced into approximately 1000 cells, conditions designed to increase signal and decrease background. Analysis of each pool is carried out in triplicate to increase confidence in the relevance of genes that drop-out in multiple replicates. Following infection, a reference sample is harvested to allow determination of

the initial level of each shRNA in the population. The cells are then selected for expression of the shRNA library and propagated for 8 population doublings, carrying enough cells to maintain the representation of the library in the population. End samples are then harvested, and genomic DNA is prepared from all samples. Once all of the pools are completed, we will generate probes for microarray from each genomic sample by amplifying barcodes built into the shRNA library vector. These probes will be labeled with Cy3 (for the end samples) or Cy5 (for the initial samples), then competitively hybridized to barcode microarrays to determine the change in representation of each shRNA over time. These data will be analyzed using the method of significance analysis of microarrays (SAM) to identify shRNAs that are consistently depleted across triplicates more than two-fold. Genes whose loss of function is synthetically lethal with loss of BRCA1 function will be defined as those targeted by shRNAs that are preferentially lost from the HCC1937-GFP cells, but are maintained in the reconstituted HCC1937-BRCA1 cells.



Figure 3. Schematic of shRNA library screen.

Characterization of BRCA1 synthetic lethal genes

The genes identified from the genome-wide screen as BSLs will be validated individually using multiple shRNAs for each gene. The primary validation assay will be a multi-color competition assay (MCA)³⁶. HCC1937-GFP cells will be infected with an inducible shRNA vector that also inducibly expresses red fluorescent protein (RFP) from the same promoter. These cells will be mixed with mock-infected HCC1937-GFP cells and treated with doxycycline to induce shRNA and RFP expression. After 24 hours of doxycycline treatment, the mixed cells will be analyzed by flow cytometry to determine the initial percentage of RFP positive (and shRNA expressing) cells. These cells will be cultured for several population doublings in doxycycline and then analyzed again by flow

cytometry to determine whether the shRNA expressing cells have decreased as a percentage of the cell population. Those genes that are found to be synthetically lethal with BRCA1 in this assay will be further characterized using gene ontology and databases of proteins that are frequently mutated or overexpressed in cancers to prioritize candidates for further study.

Key Research Accomplishments

- Generation of clonal isogenic BRCA1 wild-type and BRCA1 mutant breast cancer cell lines
- Demonstration of functionality of BRCA1 reconstitution by showing decrease in IR sensitivity
- Beginning genome-wide shRNA library screen in clonal cell lines

Reportable Outcomes

As this study is still at an early stage, no reportable outcomes have been reached.

Conclusions

Identifying novel targets for treatment of *BRCA1* mutant breast cancers has the potential to lead to vastly improved outcomes for patients with these tumors. This screen will allow us to identify and exploit weaknesses in BRCA1-deficient cells that are created by the lack of functional BRCA1 protein. As the cell lines used in this screen are isogenic apart from expression of BRCA1, we will be able to specifically identify genes that are synthetically lethal with BRCA1 mutation and not generally cell lethal. Now that we have appropriate cell lines in hand, we are moving forward with the shRNA library screening and will begin validating candidates as soon as the data is available.

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