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PRINCIPAL INVESTIGATOR: Stephen Elledge

CONTRACTING ORGANIZATION: The Brigham and Women's Hospital, Inc.
Boston, MA 02115

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14. ABSTRACT A major obstacle for breast cancer drug discovery is target identification. Despite the wealth of information on copy number alterations and mutations recently available from sequencing efforts such as The Cancer Genome Atlas, identifying new targets for breast cancer therapeutics has proved challenging because causative perturbations cannot be determined from benign changes without functional studies. Genetic shRNA screens are powerful 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 shRNA libraries in retroviral and lentiviral vectors that target the entire human genome. Our proposal aims to use this novel screening technology to identify new therapeutic targets for breast cancers harboring BRCA1 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 BSLs for BRCA1 Synthetic Lethal genes, are essential in cells lacking BRCA1, and as such are prime targets for therapeutic intervention 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 and one with a mutant BRCA1 gene. We have shown that these cells are differentially sensitive to DNA damage in the form of ionizing radiation. Cells carrying a mutant BRCA1 are more IR-sensitive than their complemented wild-type counterparts. We have begun to perform the synthetic lethal shRNA screen on these two cell lines 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

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^{1,2}. 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 have introduced 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

Genome-wide shRNA screening for *BRCA1* synthetic lethal genes

As we discussed in our last update, we have generated a pair of clonal, isogenic cell lines, HCC1937-GFP and HCC1937-*BRCA1*, for use in our screen. HCC1937 harbors a homozygous mutation of *BRCA1*, 5382insC, which results in truncation of the *BRCA1* protein and loss of one of the BRCT repeats³. HCC1937-*BRCA1* expresses a bicistronic retroviral construct that contains both wild-type *BRCA1* and green fluorescent protein (GFP), which can be used as a marker of retroviral expression⁴. HCC1937-GFP cells express a GFP-only retrovirus, and are thus still deficient in *BRCA1* protein (Figure 1). As we showed in our last update, reconstitution of HCC1937 with *BRCA1* rescued its radiation sensitivity.

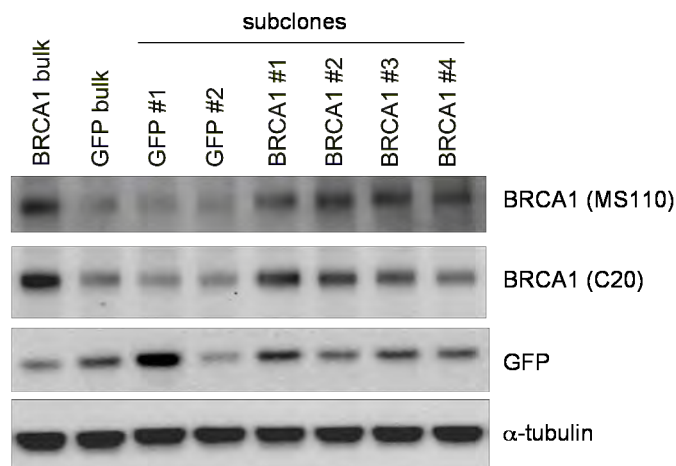


Figure 1. Expression of reconstituted wild-type *BRCA1*. Expression of *BRCA1* was analyzed in the parental bulk HCC1937-GFP and HCC1937-*BRCA1* populations, as well as in several subclones from each population. Two *BRCA1* antibodies, MS110 and C20, were used. GFP expression was also assayed. The clones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

HCC1937-GFP and HCC1937-BRCA1 were transduced with our retroviral shRNA library that targets the entire human genome (Figure 2). Our library is divided into six pools of approximately 13,000 shRNAs each. Each pool was introduced into each cell line 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 was carried out in triplicate to increase confidence in the relevance of genes that drop-out in multiple replicates. Following infection, a reference sample was harvested to allow determination of the initial level of each shRNA in the population. The cells were 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 were then harvested, and genomic DNA was prepared from all samples. We generated probes for microarray from each genomic sample by amplifying half of each shRNA. These probes were labeled with Cy3 (for the end samples) or Cy5 (for the initial samples), then competitively hybridized to microarrays to determine the change in representation of each shRNA over time. These data were analyzed using the method of significance analysis of microarrays (SAM) to identify shRNAs that were consistently depleted across triplicates in the BRCA1-deficient cell line more than two-fold. Genes whose loss of function is synthetically lethal with loss of BRCA1 function were defined as those targeted by shRNAs that are preferentially lost from the HCC1937-GFP cells, but are maintained in the reconstituted HCC1937-BRCA1 cells.

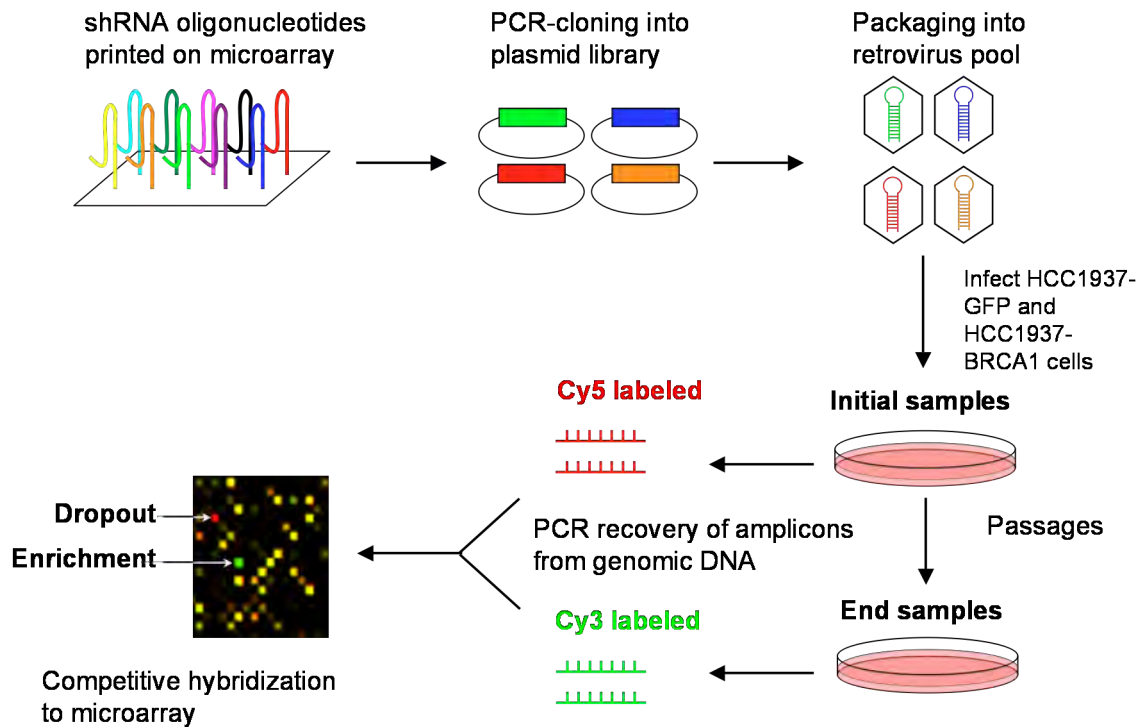
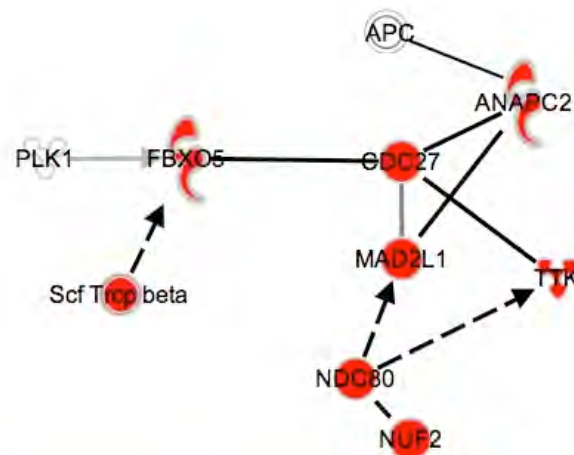


Figure 2. Schematic of shRNA library screen.

Characterization of BRCA1 synthetic lethal genes

Using the criteria outlined above, we have identified 630 shRNAs, targeting 590 genes, that scored as being BRCA1 synthetic lethal genes (BSLs). Within this large number of genes, several pathways and cellular processes were enriched, giving us a clearer picture of the types of cellular stresses that BRCA1-deficient cells are particularly sensitive to. One of the major pathways represented in the BSL list is regulation of spindle formation and entry into mitosis (Figure 3). For example, three shRNAs targeting NDC80, which is a key regulator of kinetochore-microtubule attachment and is required for proper chromosome segregation, scored in our screen as being synthetically lethal with BRCA1. Additionally, NUF2, another component of the NDC80 complex, also scored as a BSL. Two shRNAs targeting NDE1, which functions in regulation of dynein localization to kinetochores and is required for proper chromosome alignment, also were identified as synthetically lethal with BRCA1. These examples, along with other genes involved in this pathway that scored in the screen, strongly suggest that the lack of functional BRCA1 sensitizes cells to the disruption of spindle formation and highlight this pathway as a possible target for treatment of BRCA1-deficient cancers.



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Figure 3. Network of BSLs that function during spindle formation and mitosis. Ingenuity Pathway Analysis of a subset of the proteins involved in mitosis that scored as BSLs in the shRNA screen. The red shaded proteins scored in the screen; additional proteins are shown for context.

Another pathway containing multiple proteins that scored in the screen is the formation of COPI coatomer vesicles, which mediate retrograde transport from the Golgi to the endoplasmic reticulum. COPA, COPB1, COPB2, COPG and ARCN1 (delta-COP) all scored in the screen, and all are components of the COPI complex. BRCA1-deficient cells may be generally unable to deal with the additional stress of losing a major transport pathway, or there may be specific functions of COPI, such as promoting autophagy, that are critical for BRCA1-deficient cell survival⁵.

BRCA1-deficient cells are also particularly sensitive to depletion of other proteins involved in DNA repair and DNA replication. This sensitivity is already being exploited with the use of PARP1 inhibitors to treat BRCA1 tumors. Three shRNAs targeting MMS22L, which was recently described as being involved in replication stress control, scored in our screen, suggesting that BRCA1 and MMS22L may function in parallel pathways in the response to replication stress. Additional DNA damage response proteins targeted by shRNAs that scored in the screen include EXDL2, CDC5L, MDC1 and HEL308. As with PARP1, some of these proteins may provide ideal targets for treatment of BRCA1-deficient tumors, as the redundancies normally present in the DNA damage response pathways could protect BRCA1-proficient cells from harm.

The proteins and pathways mentioned here represent a few of the most promising BSL hits. These hits, along with the other BSLs identified in the screen, will now be validated with additional shRNAs to determine which are truly synthetically lethal with BRCA1 deficiency. The primary validation assay will be a multi-color competition assay (MCA)⁶. 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. We will also test these shRNAs in additional BRCA1-deficient cell lines. Once we have identified genes that provide strong synthetic lethality in multiple BRCA1-deficient cell lines, we will move on to developing ways to exploit those sensitivities in tumors.

Key Research Accomplishments

- Completion of genome-wide shRNA screen in isogenic BRCA1-deficient and reconstituted cell lines
- Identification of a cohort of BRCA1-synthetic lethal genes that are promising candidates to target in BRCA1-deficient tumors

Reportable Outcomes

We have identified a large number of genes that are potentially synthetic lethal with BRCA1, and once these have been validated they will be an important advance in our understanding of the biology of BRCA1-deficient cells and how to specifically target their sensitivities.

Conclusions

We have identified a large cohort of genes through our genome-wide shRNA screen that are potentially synthetically lethal with BRCA1. Within this cohort, several cellular processes are over-represented, suggesting that disruption of these processes is particularly detrimental to BRCA1-deficient cells. Formation of the mitotic spindle, Golgi-to-ER transport by COPI-coated vesicles and the DNA damage response are three examples of cellular processes that are targeted repeatedly in the BSL cohort and represent promising leads for development of new treatments for BRCA1-mutant cancers.

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