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**Title and Subtitle**

Understanding *B. amphibiose* c-Myc Function in Human Breast Cancer Development

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**Supplementary Notes**

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**Abstract**

My research is focused on BRCA2, whose mutation has been implicated in the development of breast, ovarian, prostate, pancreatic cancers and Fanconi anemia. BRCA2 is an extremely large protein that is challenging to study. In order to study this important tumor suppressor systematically and comprehensively, I have made a series of rabbit polyclonal antibodies against different parts of human BRCA2. These antibodies have led to the observation that BRCA2 colocalizes with BRCA1 to S-phase nuclear foci and ionizing radiation-induced foci and that BRCA2 possibly localizes to a single large nuclear structure in primary human fibroblasts during G1 phase of the cell cycle when BRCA1 expression level is low. Using a double-tagging strategy, I have also purified a BRCA2 C-terminal domain complex and identified a pair of ATPases, Tip48 and Tip49, as BRCA2 interacting proteins.
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INTRODUCTION

Breast cancer is the most frequently diagnosed cancer among women and is the No. 1 health threat to women in the United States and other western countries. One tenth of western women develop breast cancer during their lifespan. A significant proportion of breast cancer cases, in particular those arising at a young age, are clustered in certain families that have germline mutations in either one of two breast cancer susceptibility genes, BRCA1 and BRCA2. My research is focused on BRCA2, whose mutation has been implicated in the development of breast, ovarian, prostate, pancreatic cancers and Fanconi anemia. Human BRCA2 is a very large protein consisting of 3,418 amino acids, approximately twice the size of BRCA1. It is essential for embryonic development, as mice deficient of BRCA2 die before birth. It is also essential for homologous recombination and DNA repair. BRCA2 mutant cells show gross genomic instability and sweeping chromosomal abnormalities, including broken chromosomes and all forms of unnatural, illegitimate chromosomal structures. Normal cells arrest or slow down DNA synthesis after DNA damage to allow time for DNA repair, while BRCA2 deficient cells show radio resistant DNA synthesis (RDS) without efficient repair, which certainly leads to accumulation of replication errors and promote cancer formation (see Vankitaraman 2002 for review). The goal of my research is to elucidate some of the biological functions of BRCA2 through generation/utilization of new reagents, identification of its interacting proteins.

BODY (STRATEGIES AND PROGRESS)

Our understanding of BRCA2 at the molecular level is still limited so far, presumably due to its large size which makes it difficult to study and to a lack of good reagents, especially high-avidity, monospecific antibodies, that can track the protein in the cell. A protein of this size usually behaves as a master protein coordinating complex signals in multiple cellular processes by interacting and forming dynamic complexes with many other proteins. The strategies and progress of this research are as follows.

I. Tracking the cellular localization of BRCA2 through generation and utilization of new antibodies. As mentioned before, BRCA2 is an extremely large protein that is challenging to study. In order to study this important tumor suppressor systematically and comprehensively, a collection of high quality reagents are absolutely required. I have made a series of six rabbit polyclonal antibodies against different parts of human BRCA2 and I am in the process of making mouse monoclonal antibodies against the N- and C-terminus of human BRCA2. Among the six polyclonal antibodies mentioned above, two of them, anti-BRCA2F5 and anti-BRCA2F8, have turned out to be particularly valuable and enabled me the following observations:

1. BRCA2 colocalizes with BRCA1 in discrete nuclear foci (dots) in S-phase (Figure 1A to C). This has been reported by my predecessors of the Livingston lab (Chen et al., 1998). Regrettfully, due to a lack of sufficient amount of good reagents (antibodies), the project had been discontinued. With this antibody (anti-BRCA2F8) in hand, I plan to check whether BRCA2 is localized on the inactive X-chromosome as is BRCA1 (Ganesan et al., 2002). This antibody also enables me to screen for BRCA2 SiRNAs using immunofluorescence and to do many other experiments.
2. BRCA2 colocalizes with BRCA1 in nuclear dots after ionizing radiation (Figure 1D to F). BRCA2 has been reported to colocalize with proliferating cell nuclear antigen (PCNA) at sites of DNA damage after treatment of cells by hydroxyurea (HU) (Chen et al., 1998). However, an unambiguous colocalization of BRCA2 and BRCA1 following ionizing radiation has not been reported. My results clearly indicate that these two proteins collaborate at the same sites of DNA damage to facilitate DNA repair.

3. BRCA2 is possibly localized in a single large nuclear focus in primary human fibroblasts during the G1 phase (Figure 2). One of my affinity-purified polyclonal antibodies (anti-BRCA2F5) stained a large single focus in approximately 20% of both primary human fibroblasts (Figure 2A). Interestingly, this large focused staining disappeared after cells were γ-irradiated and a few hours later, the same antibody decorated a number of smaller discrete foci which gradually develop over time (Figure 2D). Also interesting is that the subpopulation of cells that contain such single large focus happened to be negative for BRCA1 staining (Figure 2B and C). As BRCA1 is expressed primarily in S and G2 phase, this observation indicates that BRCA2 may be responsible for DNA repair in G1 (or possibly G0) phase when BRCA1 is absent.

II. Using a “double-tagging” strategy to isolate BRCA2 complexes and identify its partner proteins. Dr. Yoshihiro Nakatani’s group in our department has developed a powerful system to purify protein complexes in which a protein of interest is double-tagged with FLAG and HA epitopes, introduced into Hela cells using retroviral transduction and subsequently isolated through two steps of affinity purification from extracts of large cultures (Ikura et al., 2000). Following this strategy, I have made retroviral constructs expressing human BRCA2 in three large fragments which are all double-tagged at either N or C-terminus. The reason to express BRCA2 in fragments is that full-length BRCA2 cDNA is too large to be fit into any retroviral vector.

As a first trial, I have established a Hela cell line that stably express the double-tagged BRCA2 C-terminal domain (CTD) because it is the most highly conserved part of BRCA2 proteins across species and contains many clinically relevant mutations. Structurally, this domain contains three OB folds, which suggest a role of it in BRCA2-DNA binding (Yang et al., 2002).

The FLAG-HA double-tagged CTD complexes were purified from its stable expressing cells lines in sufficient, Coomassie blue-stainable amounts (Figure 3A). Both tagged versions of CTD pulled down a similar set of protein bands. One of the bands with a molecular weight of approximately 50 KD has been analyzed by mass spectroscopy and determined to contain a pair of ATPases, Tip48 and Tip49, which are human homologues of E. coli RuvB responsible for resolving the Holiday junction formed after homologous recombination. The existence of Tip48 in the BRCA2 CTD complex was confirmed by western blot analysis (Figure 3B). More importantly, Tip48 antiserum was found to immunoprecipitate endogenous BRCA2 as well (Figure 3C), indicating that Tip48 interacts with BRCA2 under physiological condition. The biological relevance of this interaction is being investigated.

Future works in this aspect include purification of complexes of BRCA2 N-terminal domain and central domain which directly interacts with Rad51 recombinase. Identification of additional relevant BRCA2-interacting proteins will certainly shed light
on our understanding of the exact role BRCA2 plays in homologous recombination and DNA repair.

KEY RESEARCH ACCOMPLISHMENTS:

- Generated six polyclonal BRCA2 antibodies with two of them particularly useful
- Observed colocalization of BRCA2 and BRCA1 in S-phase nuclear foci
- Observed colocalization of BRCA2 and BRCA1 in ionizing radiation-induced foci
- Observed a possible localization of BRCA2 in a large nuclear focus in G1 phase
- Identified Tip48 and possible Tip49 as BRCA2 interacting protein

REPORTABLE OUTCOMES: N/A

CONCLUSIONS

During this first year of grant support, I have made significant progress. Remarkably, the generation of anti-BRCA2F5 and anti-BRCA2F8 has enabled me to see this important tumor suppressor protein in the cell with a clarity nobody has ever reportedly achieved. These great reagents will certainly open the door for more exciting discoveries in the future. In addition, I have mastered the whole process of double-tagging affinity purification method and will be able identify more BRCA2 interacting proteins, which will potentially significantly elevate our understanding of BRCA2 function.

REFERENCES


Figure 1. BRCA2 colocalizes with BRCA1 in S-phase nuclear foci (A to C) and ionizing radiation-induced nuclear foci (D to F). U2OS cells were fixed, permeabilized, and double-stained with a BRCA2 polyclonal antibody (A and D) and a BRCA1 monoclonal antibody (B and E), before or 14 hr after 15 Gy γ-irradiation. Merged images are shown in C and F. The BRCA2 antibody used (anti-F4) was raised against a GST-fusion protein containing the C-terminal region of human BRCA2 protein and affinity purified. The BRCA1 monoclonal antibody was SD118.
Figure 2. Possible localization of BRCA2 in a single large nuclear focus in primary human fibroblasts during the G1 phase. (A to C) Asynchronously growing human primary lung fibroblast IMR90 cells were double-stained with a BRCA2 polyclonal antibody (A) and BRCA1 monoclonal antibody SD118 (B). A merged image is shown in C. (D) IMR90 cells were stained with the BRCA2 polyclonal antibody 16 hr after 15Gy γ-irradiation. The BRCA2 antibody (anti-F5) used was raised against a GST-fusion protein containing the central region of the human BRCA2 protein and affinity purified.
Figure 3. BRCA2 interacts with Tip48. (A) Coomassie Blue staining of components of BRCA2 C-terminal domain (CTD) complexes purified using a double-tagging (FLAG and HA) method. Lane 1 and 2, components of complexes of BRCA2 CTD tagged either at the N-terminus (lane 2) or at the C-terminus (lane 3). (B) BRCA2 CTD interacts with Tip48. Materials bound on anti-FLAG M2 agarose from lysate of control Hela cells (lane 1) and cells stably expressing BRCA2 CTD double-tagged at the C-terminus (lane 2) were eluted using FLAG peptide, resolved by SDS-PAGE and blotted onto a nitrocellulose membrane and probed with an anti-Tip48 polyclonal antibody. (C) Coimmunoprecipitation of endogenous BRCA2 and Tip48. Whole cell extract of 293 cells was immunoprecipitated with preimmune serum (lane 1), sera from four different anti-Tip48 bleeds (lanes 2 through 5) and anti-BRCA2F2 (lane 6). BRCA2 in the precipitates was detected with a monoclonal antibody (Ab-1, Oncogen Research Products).