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The role of CtIP in BRCA1-mediated tumor suppression

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14. ABSTRACT CtIP is reported to function as a tumor suppressor; however, it is not known whether this function reflects its activity in the BRCA1 pathway and, as such, would be relevant to human breast and ovarian cancer. Indeed, since Ctip-null mice die at an earlier stage of embryogenesis than Brca1-null animals, CtIP is likely to execute a least some functions independently of BRCA1. Therefore, to study specifically the BRCA1-dependent functions of CtIP, I will examine tumor suppression and genomic stability in cells/mice that expresses a CtIP polypeptide that fails to interact with BRCA1, the CtIP-S326A mutant. To do this, I prepared knock-in targeting constructs that were designed to generate conditional-null (Ctip ^{Co}) and S326A-mutant (Ctip ^{S326A}) alleles of the mouse <i>Ctip</i> gene. These constructs were independently electroporated into 129Sv ES cells. Following selection, resistant colonies were examined by Southern analysis to identify correctly targeted clones. Several independent Ctip ^{Co/+} clones or Ctip ^{S326A/+} clones were injected into blastocysts to derive germline chimeras. Control (Ctip ^{Co/+} , Wap ^{Cre/+}) and experimental (Ctip ^{S326A/Co} , Wap ^{Cre/+}) cohorts are currently being generated to determine whether the BRCA1-CtIP interaction is required for mammary-epithelial cell specific tumor suppression. Furthermore, we obtained isogenic ES cells that are either Ctip ^{+/-} or Ctip ^{S326A/-} by targeting the wildtype allele of Ctip ^{+/-} or Ctip ^{S326A/+} ES cells with a Ctip-null allele. We are currently assessing the genomic stability function of the BRCA1-CtIP interaction by comparing the CtipS326A-mutant's ability to repair DNA double strand breaks, resist genotoxic stresses, and suppress spontaneous and damage-induced chromosomal defects to the matched wildtype cell line (Ctip ^{+/-}).				
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Introduction:

In vivo, most if not all endogenous BRCA1 polypeptides associate with the BARD1 protein to form a potent ubiquitin E3 ligase. Since the E3 ligase activity of BRCA1/BARD1 is ablated by certain tumor-associated BRCA1 missense mutations, the enzymatic function of the heterodimer is likely to be required for tumor suppression. Thus, to elucidate the molecular mechanisms of BRCA1-mediated tumor suppression, it is necessary to identify its enzymatic substrates and determine the biological consequences of their ubiquitination. Only one substrate to date, the CtIP protein, is known to play a prominent role in the maintenance of genomic stability, and as such, may serve as a key downstream mediator of BRCA1 tumor suppression. To evaluate the role of CtIP in this BRCA1 function, I am assessing whether introducing a missense mutation that disrupts the molecular interaction between BRCA1 and CtIP leads to tumorigenesis in mammary epithelial cells. A cre/lox gene targeting system was used to generate mice that disrupt the Brca1-Ctip interaction in a mammary-specific fashion. Additionally, I am assessing the genomic stability functions of the BRCA1-CtIP interaction by comparing isogenic cells that express either wildtype CtIP or the S326A-CtIP mutant.

Body:

Given its pivotal role in the DNA damage response, CtIP may serve as a key mediator of BRCA1 tumor suppression. To determine whether CtIP is required for BRCA1-mediated tumor suppression (Task 1), I prepared a targeting construct to introduce the S326A mutation into ES cells. Previous studies have established that the *in vivo* association of human CtIP and BRCA1 requires phosphorylation of CtIP at residue S327, which corresponds to S326 in mice [1]. Therefore, the BRCA1-CtIP interaction is disrupted in cells that express only the S326A allele of Ctip. This construct has the S326A mutation introduced into the coding sequence of exon 11, as well as a LoxP-flanked neomycin-resistant gene cassette in the upstream intron. After electroporation of 129Sv ES cells with this construct, neomycin-resistant colonies were selected, properly recombined clones were identified by Southern analysis, and the presence of the S326A mutation was confirmed by sequence analysis. The neomycin gene cassette was removed by transiently transfecting in a plasmid encoding Cre recombinase. Thus, Ctip^{S326A/+} ES clones have been obtained. Two independent clones have recently been injected into blastocysts to derive germline chimeras and the heterozygous knock-in mice are currently being bred to generate animals that solely express the mutant Ctip-S326A polypeptide (i.e., Ctip^{S326A/-} and Ctip^{S326A/S326A} mice). I will then assess the development of these animals using the same parameters used previously to characterize the phenotypes of mice bearing null alleles of Ctip, Brca1, and Bard1 [2, 3, 4]. The phenotype of the Ctip-S326A mutant mice will reveal the importance of the BRCA1 interaction for CtIP functions.

Additionally, a targeting vector was constructed to generate the Ctip conditional-null allele (Ctip^{Co}). Using a similar approach as the S326A-mutant, heterozygous Ctip^{Co/+} ES cells were generated and the conditional-null allele was introduced into the mouse germline. Matings are currently in progress to more specifically examine the role of the BRCA1-CtIP interaction in suppression of basal-like breast cancer by analysis of tumor development in a cohort of conditional Ctip-S326A mutant females (Ctip^{S326A/Co}, Wap^{Cre/+}). These mice will be mated to induce pregnancy, lactation, Cre expression, and mammary-

specific recombination of the conditional $Ctip^{Co}$ allele. Tumor formation in this cohort will be monitored and compared to that of the female control cohort ($Ctip^{Co/+}$, $Wap^{Cre/+}$) and the conditional $Brca1$ -null females ($Brca1^{flex2/-}$, $Wap^{Cre/+}$), which develop basal-like invasive mammary carcinomas with a latency (T_{50}) of approximately 500 days [5].

Isogenic cell lines will be used to address the genomic stability functions of the BRCA1-CtIP interaction (Task 2). As mentioned above, $Ctip^{S326A/+}$ ES cells were generated by targeting the $Ctip^{+/+}$ ES cells with the point mutant targeting construct. Furthermore, the $Ctip^{S326A/+}$ ES cells were targeted with a $Ctip$ -null allele in order to make $Ctip^{S326A/-}$ ES cells. Since $Ctip$ -null ES cells are not viable, our ability to generate $Ctip^{S326A/-}$ cells suggests that the BRCA1 interaction is not required for all CtIP functions. Standard assays are currently being employed to ascertain whether double strand break repair, cellular resistance to genotoxic stress, and suppression of spontaneous and damage-induced chromosomal defects are dependent on the BRCA1-CtIP interaction.

Key Research Accomplishments:

- Generated a $Ctip$ conditional-null targeting construct
- Cloned the $Ctip$ S326A-mutant targeting construct which disrupts the BRCA1-CtIP interaction
- Obtained heterozygous $Ctip^{Co/+}$ ES cells, as well as, $Ctip^{S326A/+}$ ES cells
- Derived both $Ctip^{Co/+}$ and $Ctip^{S326A/+}$ germline chimera mice
- Targeted the wildtype allele of $Ctip^{+/+}$ and $Ctip^{S326A/+}$ ES cells with the $Ctip$ -null allele to generate isogenic cell lines that are either $Ctip^{+/-}$ or $Ctip^{S326A/-}$

Reportable Outcomes:

In determining whether the CtIP protein is required for BRCA1-mediated tumor suppression, I have successfully generated two targeting constructs, the $Ctip$ conditional-null construct and the $Ctip$ -S326A mutant construct. With the conditional-null construct, CtIP is inactivated following Cre recombinase expression. The S326A-mutant construct, on the other hand, has a missense mutation in exon 11 preventing phosphorylation of CtIP and binding to BRCA1. Independently, these targeting constructs were electroporated into $Ctip^{+/+}$ 129Sv ES cells to generate several independent $Ctip^{Co/+}$ and $Ctip^{S326A/+}$ ES clones. These clones were identified by Southern blotting and confirmed by nucleotide sequence analysis. Several independent $Ctip^{Co/+}$ clones or $Ctip^{S326A/+}$ clones were injected into blastocysts to obtain germline-transformed mice bearing either the conditional-null $Ctip^{Co}$ allele or the S326A-mutant allele $Ctip^{S326A}$. Eventually, $Ctip^{S326A/+}$, $Wap^{Cre/+}$ animals will be crossed with $Ctip^{Co/Co}$ animals to yield experimental ($Ctip^{S326A/Co}$, $Wap^{Cre/+}$) and control ($Ctip^{Co/+}$, $Wap^{Cre/+}$) female mice that will be mated to induce pregnancy, lactation, Cre expression, and mammary-specific recombination of the conditional $Ctip^{Co}$ allele. Tumor formation will be monitored in these cohorts and compared to that of conditional $Brca1$ -null females ($Brca1^{flex2/-}$, $Wap^{Cre/+}$).

Additionally, the $Ctip^{S326A/+}$ ES clones generated in the process of introducing this mutation into the mouse germline were targeted with the $Ctip$ -null allele in an attempt to make $Ctip^{S326A/-}$ ES cells. These cells were easily obtainable suggesting that the BRCA1-CtIP interaction is not required for ES cell viability, despite the fact that both BRCA1 and CtIP are individually required for viability [2, 4]. A variety of assays to assess the genomic

stability functions of the BRCA1-CtIP interaction are underway, including assays that compare the ability of CtIP^{+/-} and CtIP^{S326A/-} ES cells to repair double strand breaks, resist several genotoxic stresses, and suppress spontaneous and damage-induced chromosomal aberrations.

Conclusion:

To date, much of my research has involved generating mice and ES cells bearing either the CtIP^{Co} allele or the CtIP^{S326A} allele. Through this process, we have learned that CtIP^{S326A/-} ES cells are viable, and thus, the BRCA1-CtIP interaction is not required for ES cell viability. Since our isogenic cell panel that expresses either wildtype CtIP or the S326A-mutant CtIP is in place, we can efficiently and properly assess which functions of CtIP, if any, are dependent on the BRCA1-CtIP interaction. The fact that these ES cells are viable indicates that the BRCA1-CtIP interaction is not required for all functions of CtIP. Furthermore, we will soon generate the experimental and control cohorts of mouse necessary to ascertain whether the BRCA1-CtIP interaction is required for BRCA1-mediated tumor suppression. If the experimental (CtIP^{S326A/Co}, Wap^{Cre/+}) females develop breast tumors, we will conclude that the BRCA1-CtIP interaction is required for tumor suppression in mammary epithelial cells. The latency and penetrance of tumor development compared to the conditional Brca1-null mice will further elucidate the requirement of the BRCA1-CtIP interaction in the tumor suppression activity of BRCA1. Through this complete analysis, using both ES cells and mice, we can learn what role the BRCA1-CtIP interaction has in maintaining genomic stability and in suppressing tumor formation.

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