AD_____

Award Number: W81XWH-04-1-0325

TITLE: A Mouse Model to Investigate the Role of DBC2 in Breast Cancer

PRINCIPAL INVESTIGATOR: Valerie Boka

CONTRACTING ORGANIZATION: University of Texas Health Science Center
San Antonio, Texas 78229-3900

REPORT DATE: March 2007

TYPE OF REPORT: Annual Summary

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-03-2007	Annual Summary	15 Feb 2004 – 14 Feb 2007

4. TITLE AND SUBTITLE

A Mouse Model to Investigate the Role of DBC2 in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0325

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Valerie Boka

E-Mail: boka@uthscsa.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

University of Texas Health Science Center
San Antonio, Texas 78229-3900

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
Sporadic breast cancer represents 90% of breast cancer patients. Mutations of both oncogenes and tumor suppressor genes often occur in spontaneous breast cancer. Specifically, tumor suppressor gene activity may be abrogated or decreased in cancer cells. Recently, a putative tumor suppressor gene, DBC2 (Deleted in Breast Cancer), was discovered that appears to be frequently mutated in sporadic breast cancer. DBC2 is suspected to be a tumor suppressor gene important for breast cancer because: 1) DBC2 expression cannot be detected in half of the spontaneous breast cancer tissues and cells tested and 2) wild-type (WT) DBC2 expressed in a breast cancer cell line, T47D, inhibited cellular proliferation while mutated DBC2 expression did not repress growth of the breast cancer cells. These data imply that mutation of DBC2 is important for the development of spontaneous breast cancer. This work serves ton investigate the functional role of DBC2 in cells and mice to elucidate the function of DBC2 for tumor suppression. To this end, we have begun targeting the DBC2 allele and are constructing vectors that will express wildtype human DBC2 cDNA and altered cDNAs.

15. SUBJECT TERMS
Deleted in breast cancer, DBC2, spontaneous breast cancer

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	9	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

A Mouse Model to Investigate the Role of *DBC2* in Breast Cancer

INTRODUCTION

Breast cancer is a major cause of mortality among women. The American Cancer Society predicts that about one in eight women will be diagnosed with breast cancer [1]. Deletion of tumor suppressor genes play an important role in both familial and sporadic breast cancer [2]. Hereditary breast cancer is frequently due to a germline heterozygous mutation of either Brca1 or Brca2 [3]. Familial breast cancer represents only 10% of total breast cancer cases; and Brca2 is not mutated for spontaneous breast cancer; thus, the etiology for 90% of breast cancer is largely unknown. However, Hamaguchi and colleagues recently discovered a putative tumor suppressor gene, *DBC2* (deleted in breast cancer), that appears to be frequently mutated in sporadic breast cancer [4]. The *DBC2* gene lies within a region of human chromosome 8p21. The putative functional domains of *DBC2* include a RAS domain and two protein-protein interaction domains called the BTB/POZ domains. *DBC2* is suspected to be a tumor suppressor gene important for breast cancer because: 1) *DBC2* expression cannot be detected in half of the spontaneous breast cancer tissues and cells tested and 2) wild-type (WT) *DBC2* expressed in a breast cancer cell line, T47D, inhibited cellular proliferation while mutated *DBC2* expression did not repress growth of the breast cancer cells. These data imply that mutation of *DBC2* is important for the development of spontaneous breast cancer. Recent microarray data shows *DBC2* to have an influence on the following pathways: cell-cycle, apoptosis, cytoskeleton, and membrane-trafficking; suggesting a role for *DBC2* in carcinogenesis [5]. Furthermore, Wilkins et al. proposed a model in which *DBC2* may function as a tumor suppressor by facilitating the recruitment of proteins and their subsequent degradation by the Cul3 ubiquitin ligase complex [6]. However, due to the fact that *DBC2* was just recently discovered, little is known about its function. This work serves to investigate the functional role of *DBC2* in cells and mice to elucidate the function of *DBC2* for tumor suppression.

BODY

Specific Aim 1: Characterize the phenotype of *DBC2*-mutant mouse ES cell and MEF clones

a) Generate *DBC2*-mutant ES cells

b) Generate knock-ins of wild type and altered *DBC2* cDNAs in ES cells
c) Perform a genotoxic screen on wild type and knock-in ES cells
d) Analyze cell cycle checkpoints and apoptosis in knockout MEF

During the first year of the award period, I constructed a targeting vector to mutate *DBC2* in ES cells. Importantly, a positive selection cassette replaced the initiating ATG and some surrounding sequence. The vector was electroporated into ES cells, AB2.2 (isogenic wild-type) and *msh2*$^{-/-}$. Upon selection I successfully obtained three targeted clones in the *msh2*$^{-/-}$ background (Fig. 1). Unfortunately, I was unable to generate AB2.2 ES cells targeted for *DBC2* which may be due to homeology between the genomic sequence and the targeting vector. Msh2 suppresses homeologous recombination thereby reducing gene targeting in AB2.2 ES cells [7-9].

Figure 1. Ethidium Bromide stained agarose gel; 676bp product represents wild-type *DBC2*; 602bp product is the targeted mutant allele.

Also shown in Fig. 2 is the screening strategy that utilizes RT-PCR. To our knowledge, this was the first time such a method had been used to screen for targeted ES cell clones. This analysis included extracting RNA from the potential targeted ES cell cones, the generation of cDNA, and PCR amplification of the region of interest. Fig. 2 depicts the location of the primers used to amplify both wild-type and mutant *DBC2*. The forward primer used to amplify the wild-type band was situated in exon 1 containing the initiation ATG, the region deleted in the mutant allele (Fig. 2B). Alternatively, the mutant forward primer was positioned 3' to the CMV promoter of the selection cassette in an artificial exon (Fig. 2C). Therefore, this primer should only anneal in a clone targeted for *DBC2*. Both the wild-type and mutant alleles utilized the same reverse primer located in a downstream exon.

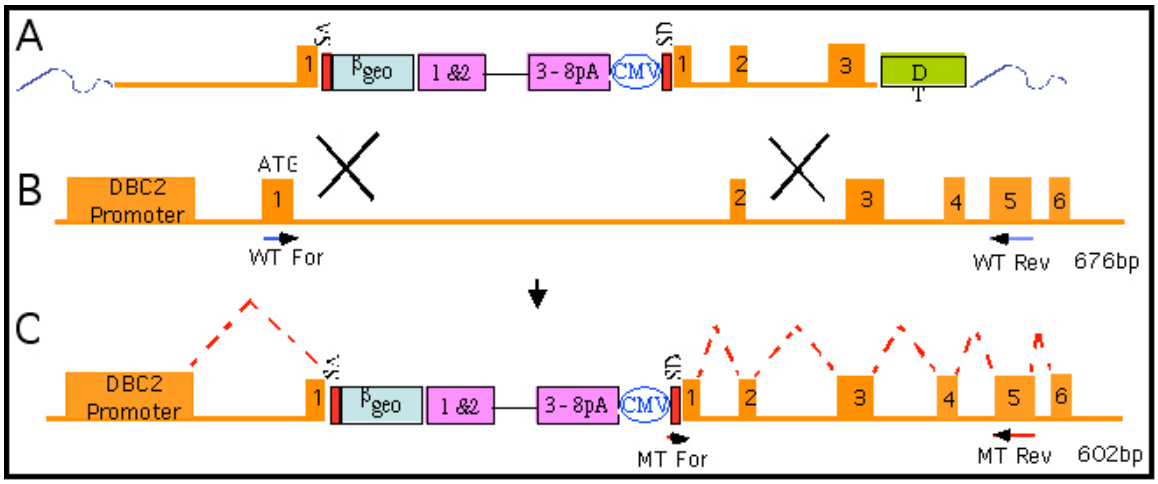


Figure 2. A. Gene targeting vector: deletes the translation initiation ATG with a promoterless Neo and an HPRT minigene. **B**. *DBC2* genomic locus. Six exons are shown. The rectangle labeled *DBC2* promoter is

undefined and presumed to be located 5' to the exon that contains the translation initiation ATG. WT For and WT Rev show the location of the primers used to identify wildtype *DBC2* by RT-PCR screening. **C**. Insertion of selection cassette into *DBC2* gene. Part of exon 1 is deleted such that the selection cassette replaces the translation initiation ATG. MT For and MT Rev show the location of the primers used to identify the mutant *DBC2* by RT-PCR (Fig. 2B,C)

.

Furthermore, the amplified sequence from the potential target was sequenced to confirm targeting using the MT For and MT Rev primers. The sequencing results showed the allele was targeted.

In subsequent years, I constructed another targeting vector to mutate *DBC2* in ES cells. A high fidelity polymerase was used to generate the targeting vector in an attempt to eliminate mismatches in the PCR product. Importantly, a positive selection cassette replaced the initiating ATG and some surrounding sequence. This targeting vector was similar to the vector generated the first year with improvements. The *HPRT* (hypoxanthine phosphoribosyltransferase) minigene is commonly used for selection of transfected clones and is unique compared to other selection cassettes in that it's coding sequence is interrupted by splicing sequences and a small intron such that exons 1&2 are separated from exons 3-8 [10]. The advantage in using the *HPRT* minigene compared to other cassettes is that one may select for both the presence and absence of HPRT in HAT (hypoxanthine, aminopterin, thymidine) and 6-thioguanine, respectively. However, the affect these splicing sequences within this minigene might have on splicing at the target gene has not been investigated. It is possible that splicing is restricted to the *HPRT* minigene; however, it is also possible that the splice acceptor (SA) preceding *HPRT* exons 3-8 can trap upstream exons in the target gene similar to promoter trap selection cassettes that have been used to randomly disrupt and identify genes [11]. It is also possible that the PGK promoter of the *HPRT* minigene can express a transcript that starts from *HPRT* exons 1&2, but then skips *HPRT* exons 3-8, and instead splices into the SA of downstream exons from the target gene. Either possibility would result in transcripts that may code for proteins and impact phenotype in unpredictable ways. In order to prevent aberrant splicing events and potentially undesired proteins that could cause unwanted phenotypes, the HPRT minigene should be removed by site-directed recombination. In the new targeting vector, the selection cassette is flanked by loxP sites that can recombine with each other upon expression of Cre recombinase as shown in figure 3. Importantly, the same selection cassette can then be used to target the second allele of *DBC2*.

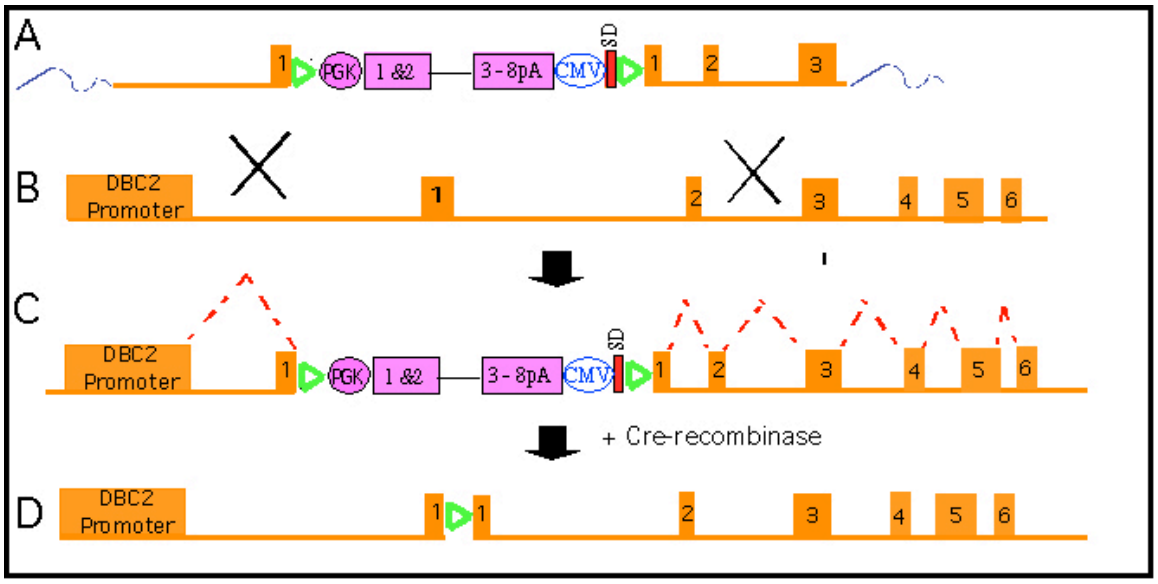
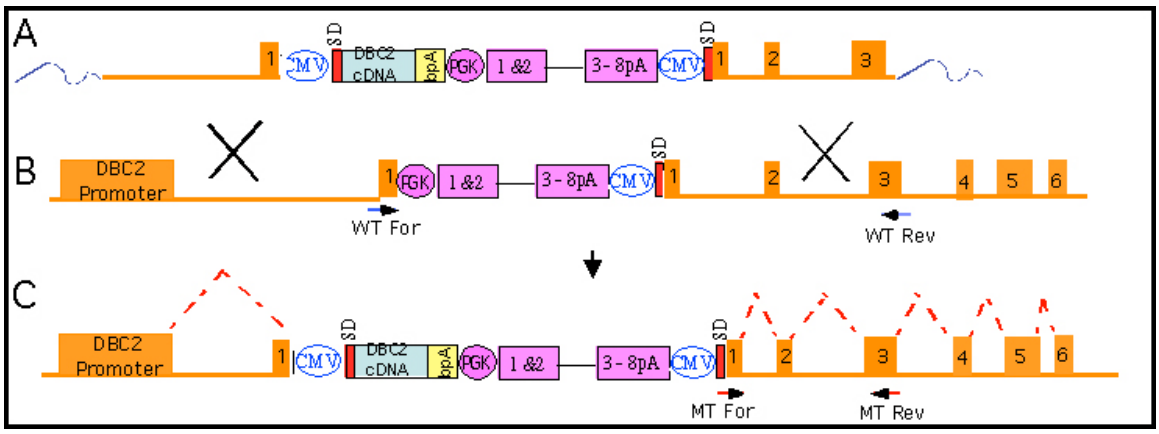


Figure 3. **A**. Gene targeting vector: deletes the translation initiation ATG with an HPRT minigene. **B**. *DBC2* genomic locus. Six exons are shown. The rectangle labeled *DBC2* promoter is undefined and presumed to be located 5' to the exon that contains the translation initiation ATG. **C**. Insertion of selection cassette into *DBC2* gene. Part of exon 1 is deleted such that the selection cassette replaces the translation initiation ATG. **D**. Removal of selection cassette from DBC2 gene. LoxP sites recombine with each other upon addition of Cre-recombinase deleting selection cassette.

The vector was electroporated into ES cells and I am currently screening for positive clones using the RT-PCR method previously described above. It is imperative to knockout *DBC2* in AB2.2 wildtype cells as we do not know the impact the absence of MSH2 will have on a DBC2-mutant phenotype.

I also began constructing the vector shown in figure 4 designed to facilitate the introduction of cDNA sequences adjacent to the endogenous DBC2 promoter. This vector utilizes homology from the *HPRT* minigene as well as from the endogenous gene to facilitate homologous recombination between the vector and a targeted *DBC2* allele in ES cells. The resulting target allows the cDNA to be expressed by the endogenous *DBC2* promoter. I have successfully amplified and started cloning human *DBC2* cDNA. Once the vector containing wild-type human cDNA is obtained, I will begin making altered *DBC2* cDNAs be introduced adjacent to the genomic *DBC2* promoter. For the altered cDNAs, I will introduce the fragment of cDNA that codes for only the BTB/POZ protein-protein interaction domains. These domains may be dominate-negative. I will also introduce cDNAs mutated for the RAS domain and cDNAs that contain mutations found in breast cancer [4] including the Asp-299 ▸ Asn mutation in the BTB/POZ domain and the Phe-647 ▸ Thr mutation in exon 9 (these two mutations likely inactivate function).

Figure 4. **A**. Gene targeting vector containing Wild type human *DBC2* cDNA. **B**. Targeted DBC2 genomic locus; *HPRT* minigene deletes translation ATG. **C**. Insertion of cDNA/selection cassette into DBC2 gene; cDNA/selection cassette replace existing selection cassette; cDNA adjacent to endogenous promoter.



Specific Aim 2: Generate and analyze *DBC2* knockout mice

DBC2 knockout mice will be generated and analyzed throughout their entire life span with particular attention given to cancer onset, incidence and spectrum. In addition, in order to investigate any potential tumor suppressor function *DBC2* has for mammary carcinoma in mice, the *DBC2*-mutation will be crossed into transgenic mice predisposed to mammary carcinoma that contain a *neu* proto-oncogene and a dominant negative p53 transgene. The mutant mice will be studied in cohorts of 30 mice (30 *DBC2*+/+, 30 *DBC2*+/-, and 30 *DBC2*-/-).

We will prepare the $DBC2^{+/-}$ ES cells for injection into blastocysts as soon as we obtain targets.

KEY RESEARCH ACCOMPLISHMENTS

- Generation of $DBC2^{+/-}$ $msh2^{-/-}$ ES cells
- Development of RT-PCR assay to detect targeted ES cells
- Amplified human DBC2 cDNA

REPORTABLE OUTCOMES

Abstracts:

None.

Manuscripts:

None

Awards:

None.

CONCLUSIONS

During the award period we attempted to target the *DBC2* gene in mouse embryonic stem cells and are currently screening for targets using the RT-PCR screening approach described previously. Additionally, we have amplified human *DBC2* cDNA and began generating a vector to rescue *DBC2*-mutant cells. Moreover, we will soon begin making altered *DBC2* cDNAs. We will perform a genotoxic screen on wild-type and mutant cells altered for *DBC2* to help elucidate a function for *DBC2* in ES cells by

testing multiple pathways important for chromosomal metabolism and responses to DNA alterations. Our studies will address the novel functional activity of *DBC2* that could have a large impact in our understanding of spontaneous breast cancer development. It is our hope that these studies will help to understand the putative activity of *DBC2* in the cell and elucidate the importance of *DBC2* as a tumor suppressor. This discovery could open many new doors for the development and implementation of drugs for the treatment of breast cancer.

REFERENCES

1. Jemal, A., et al., *Cancer statistics, 2003*. CA Cancer J Clin, 2003. **53**(1): p. 5-26.
2. Balmain, A., J. Gray, and B. Ponder, *The genetics and genomics of cancer*. Nat Genet, 2003. **33 Suppl**: p. 238-44.
3. Keen, J.C. and N.E. Davidson, *The biology of breast carcinoma*. Cancer, 2003. **97**(3 Suppl): p. 825-33.
4. Hamaguchi, M., et al., *DBC2, a candidate for a tumor suppressor gene involved in breast cancer*. Proc Natl Acad Sci U S A, 2002. **99**(21): p. 13647-52.
5. Siripurapu, V., et al., *DBC2 significantly influences cell-cycle, apoptosis, cytoskeleton and membrane-trafficking pathways*. J Mol Biol, 2005. **346**(1): p. 83-9.
6. Wilkins, A., Q. Ping, and C.L. Carpenter, *RhoBTB2 is a substrate of the mammalian Cul3 ubiquitin ligase complex*. Genes Dev, 2004. **18**(8): p. 856-61.
7. Datta, A., et al., *Mitotic crossovers between diverged sequences are regulated by mismatch repair proteins in Saccaromyces cerevisiae*. Mol Cell Biol, 1996. **16**(3): p. 1085-93.
8. de Wind, N., et al., *Inactivation of the mouse Msh2 gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer*. Cell, 1995. **82**(2): p. 321-30.
9. Selva, E.M., et al., *Mismatch correction acts as a barrier to homeologous recombination in Saccharomyces cerevisiae*. Genetics, 1995. **139**(3): p. 1175-88.
10. Reid, L.H., et al., *Regulatory elements in the introns of the human HPRT gene are necessary for its expression in embryonic stem cells*. Proc Natl Acad Sci U S A, 1990. **87**(11): p. 4299-303.
11. Friedrich, G. and P. Soriano, *Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice*. Genes Dev, 1991. 5(9): p. 1513-23.