Award Number: W81XWH-06-1-0458

TITLE: Characterizing the Role of 1p36 Deletion in Breast Cancer and Identifying Candidate Tumor Suppressors

PRINCIPAL INVESTIGATOR: Christopher S Hackett

CONTRACTING ORGANIZATION: University of California
San Francisco, CA 94143

REPORT DATE: April 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

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
4. TITLE AND SUBTITLE
Characterizing the Role of 1p36 Deletion in Breast Cancer and Identifying Candidate Tumor Suppressors

6. AUTHOR(S)
Christopher S Hackett

E-Mail: chris.hackett@ucsf.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California
San Francisco, CA 94143

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

14. ABSTRACT: Over 60% of human breast tumors display a deletion of one copy of the 1p36 region of the short arm of chromosome 1. Tumors with this deletion show a three-fold increase in mortality, suggesting a biological role for this deletion in tumor development, and suggesting the presence of one or more tumor suppressors in this region. Purpose: Characterization of the unique biology of tumors with 1p36 deletion, and characterization of the tumor suppressor(s) in the region may inform therapeutic strategies, and present unique therapeutic targets for this subset of breast cancer cases with relatively poor survival. Scope: The goals of this research project are to 1) develop a mouse model for 1p36 deletion in breast cancer by generating mice harboring loxP sequences flanking the deletion region, and crossing to tissue-specific Cre expressing mice, 2) perform in-vivo insertional mutagenesis in breast tumors using the two-component Sleeping Beauty transposon system (mutagenic transposons mobilized by a trans-acting transposase) to tag tumor suppressors and oncogenes during tumor development and 3) to combine these two systems to identify tumor suppressors in the 1p36 region. To date, we have acquired targeting constructs, generated cohorts of mice for insertional mutagenesis, and developed in vitro alternative approaches.

15. SUBJECT TERMS
1p36, insertional mutagenesis, sleeping beauty, mouse models, tumor suppressors

16. SECURITY CLASSIFICATION OF:
a. REPORT U
b. ABSTRACT U
c. THIS PAGE U

17. LIMITATION OF ABSTRACT
UU

18. NUMBER OF PAGES
11

19. NAME OF RESPONSIBLE PERSON
USAMRMC

19B. TELEPHONE NUMBER (include area code)
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Body</td>
<td>4</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>9</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>9</td>
</tr>
<tr>
<td>Conclusion</td>
<td>10</td>
</tr>
<tr>
<td>References</td>
<td>10</td>
</tr>
<tr>
<td>Appendices</td>
<td>11</td>
</tr>
</tbody>
</table>
**ANNUAL STATUS REPORT:** BC050930 Characterizing the Role of 1p36 Deletion in Breast Cancer and Identifying Candidate Tumor Suppressors

**INTRODUCTION:** Subject: Over 60% of human breast tumors display a deletion of one copy of the 1p36 region of the short arm of chromosome 1. Tumors with this deletion show a three-fold increase in mortality, suggesting a biological role for this deletion in tumor development, and suggesting the presence of one or more tumor suppressors in this region. Purpose: Characterization of the unique biology of tumors with 1p36 deletion, and characterization of the tumor suppressor(s) in the region may inform therapeutic strategies, and present unique therapeutic targets for this subset of breast cancer cases with relatively poor survival. Scope: The goals of this research project are to 1) develop a mouse model for 1p36 deletion in breast cancer by generating mice harboring loxP sequences flanking the deletion region, and crossing to tissue-specific Cre expressing mice, 2) perform in-vivo insertional mutagenesis in breast tumors using the two-component Sleeping Beauty transposon system (mutagenic transposons mobilized by a trans-acting transposase) to tag tumor suppressors and oncogenes during tumor development and 3) to combine these two systems to identify tumor suppressors in the 1p36 region. To date, we have acquired targeting constructs, generated cohorts of mice for insertional mutagenesis, and developed in vitro alternative approaches.

**BODY:** Results from all Tasks with goals in months 1-12 (and other tasks from which we have outcomes to date) from the approved Statement of Work are summarized below:

**Task 1:** Development of a mouse model for 1p36 deletion

A: Obtain and verify MICER loxP targeting clones flanking syntenic region of chromosome 4 corresponding to 1p36: We have acquired a set of 16 MICER loxP genomic targeting constructs. We have verified that 7 of these clones map to the expected region of mouse chromosome 4 (clones MHPN-295o18, MHPN-228j15, MHPN388j16, MHPN-41e21, MHPP-415n18, MHPP-4i24, MHPP352D12). Notably, in more recent assemblies of the mouse genome, the structure of the telomeric region of chromosome 4 has changed (with some sequence now mapping to other regions of the genome). To verify that the MICER clones we are using actually correspond to regions of chromosome 4, we have sent the clones to Robert Jenkins at the Mayo Clinic in Rochester, MN, to perform FISH mapping. We are awaiting the results of this FISH mapping before we begin targeting ES cells.

Since initiation of this award, another group has published a study in which they constitutively deleted a 4 Mb region of

![Figure 1: Modified 3’HPRT-V5-FRT targeting vector.](image) The modified and original vectors were cut with the HincII restriction enzyme, which has sites flanking the C-terminus of the HPRT coding region. The gel illustrates that roughly 120bp of sequence have been added to this fragment, representing the addition of a V5 epitope tag, a NotI restriction site, an FRT recognition site, and relevant spacer sequence.
mouse chromosome 4 within the 1p36 syntenic region (Bagchi, et al, 2007). While this study led to the identification of a tumor suppressor, CHD5, in the region, mice harboring this deletion did not develop breast tumors (though they did develop a range of other tumors). Similar to other candidate tumor suppressors identified in the region, this study suggests that the biology of 1p36 deletion may be complex, requiring the deletion of multiple genes. Thus, we have modified our approach in two ways in light of this new development. First, we will focus primarily on use of SB to identify specific genes on 1p36, to assess their role in breast cancer development. Second, we have modified Aim 1 to knock out a larger, 60Mb region of mouse chromosome 4, corresponding to all of the human 1p sequence present on mouse chromosome 4, to maximize our chances of capturing the biology of this deletion in human tumors. MICER targeting constructs corresponding to this deletion are among the group currently being characterized. Of course, the change in prioritization and in the scope of Aim 1 will alter our tasks such that we will prioritize characterization of tumors in SB+ mice, and will push back the timeline for characterization of mice with the 1p deletion.

Additionally, in our proposal, we noted the need for a marker to indicate the success of recombination to generate the genomic deletion in-vivo, and we proposed to use a modified 3’HPRT targeting vector with a green fluorescent reporter (IRES GFP). We have tested this vector in vitro and found that the GFP is not expressed upon recombination, as detected by flow cytometry (not shown). We have thus used overlap-extension insert PCR to engineer a new vector containing a V5 epitope tag at the end of the 3’HPRT gene sequence, followed by a FRT Flip recombinase recognition site (Fig 1). This vector will allow immediate identification of the deletion via immunohistochemistry and Western Blot using an antibody against the unique V5 epitope sequence appended to the C-terminus of the HPRT gene. The introduction of the FRT site and a new NotI restriction site will allow for easy subsequent modification of the vector in vitro or in vivo. We are currently testing expression of the epitope tag following Cre treatment in ES cell lines.

B: Transfect and screen ES cells for double-targeting in cis: Due to the questionable genomic localization of our targeting vectors, and the need to modify the targeting construct with a V5 epitope tag, we have not performed the ES cell targeting to date.
BC050930  Hackett, Christopher S.

C: Generate chimeric mice from ES cells and assay for germline transmission: As in Task 1B, we have not yet performed targeting or generated chimeric mice.

D: Breed multiply-homozygous SB, transposase, MMTV-Cre, and MMTV-Her2 mice for Aims 2 and 3: We are maintaining lines of T2/Onc, MMTV-Cre, MMTV-HER2(neu), CAGGS-SB10, RosaSB11, and Rosa-lsl-SB11;T2/Onc2 double-homozygote mice in our colony. We are in the process of generating MMTV-HER2;MMTV-Cre doubly-homozygous mice.

The Sleeping Beauty

insertional mutagenesis system works via a two part mechanism: 1) a transposon (T2/Onc), a DNA sequence capable of activating or deactivating surrounding host genes, and 2) a transposase protein (SB10 or SB11) which excises the T2/Onc transposon and re-inserts it into a random location in the host genome. Cells harboring insertions conferring tumorigenic characteristics are selected and clonally expanded, allowing for identification of the insertion site and nearby genes.

To take advantage of this system, we are generating a cohort of triply-transgenic MMTV-HER2;CAGGS-SB10;T2-Onc mice to test the mobilization of SB transposons in breast tumors (we are currently following 13 female mice, with equally-sized control cohorts lacking either CAGGS-SB10 or T2-Onc, and generating additional triply-transgenic mice). This cohort will serve two purposes: 1) to demonstrate that SB transposons are mobile in breast tumors and 2) to facilitate an initial insertional mutagenesis screen. Insertion sites mapped in these mice are likely to have direct relevance to breast cancer development.

However, since starting work on this proposal, we have learned from our collaborator David Largaespada that the expression of SB transposase in the CAGGS-SB10 mice (initially thought to be ubiquitous) is limited to muscle tissue. We have isolated protein from tumors generated in MMTV-HER2;CAGGS-SB10 mice. A Western blot for the SB transposase did not show expression of the SB10 transposase in these tissues (Fig 2). However, preliminary IHC analysis suggested that the construct may be active in advanced tumors.
While we will continue to monitor the cohort of mice with the CAGGS-SB10 construct, we will in parallel pursue the alternatives presented in our proposal. **First,** we have imported the RosaSB11 mice, which express SB in all tissues. When crossed to low-copy transposon lines, these mice develop leukemia after 4 months. We have crossed these mice to doubly-transgenic MMTV-HER2;T2-Onc (low copy) mice. If SB mobilization significantly reduces the breast tumor latency in these mice, we should be able to obtain tumor samples and identify integration sites before these mice succumb to leukemia. **Second,** to overcome the leukemia limitation with the RosaSB11 mice, we have imported the Rosa-lsl-SB11 mice into our colony. In these mice, high-level expression of SB transposase is activated via Cre-induced excision of an upstream stop sequence. We are currently breeding doubly-homozygous Rosa-lsl-SB11;T2-Onc2(high-copy) mice to MMTV-Cre and MMTV-HER2;MMTV-Cre mice. These mice should activate SB transposase in breast tissue exclusively. **Third,** we have cloned the SB transposase into three viral vectors, for in-vitro delivery of the transposase. We have cloned the SB11 transposase into 1) the pMSCV-IRE-GFP vector (pMIG-SB), an MLV-based retroviral vector with a GFP reporter, 2) the pMSCV-puro vector (pMP-SB), an MLV-based vector with a puromycin selection cassette, and 3) the pWPI lentiviral vector (pWPI-SB), an HIV-based lentivirus capable of infecting non-dividing cells (unlike MLV), and carrying a GFP reporter. We have confirmed by Western blot that all of these vectors express SB in 293T cells (Fig 3).

![Figure 4: Transduction of retroviral SB vectors into tumor cells from MMTV-HER2;T2-One mice. A: GFP expression of cells transduced with pMIG-SB 48hrs post transduction. B: Phase-contrast of the same field, showing morphology of mammary tumor cells.](image)

We have isolated tumor cells from tumors generated in MMTV-HER2;T2-Onc mice (with “dormant” transposons) and transduced the viral SB constructs in-vitro (Figure 4). We are in the process of transplanting these tumor cells into 3-week old MMTV-HER2 mice. We will monitor tumor formation via GFP reporters in two of the constructs. We will then isolate tumor tissue, isolate DNA, and assess SB transposon mobility. Should this approach work, we may also provide Cre in a similar manner in combination or in parallel; thus, we would ultimately need to generate mice carrying the floxed 1p36 allele, transposons, and MMTV-HER2 to generate the combination of deletion and mobile transposons proposed.

Additionally, in our original proposal, we suggested that a transposon concatemer on chromosome 4 would facilitate identification of tumor suppressors in the 1p36 syntenic region on mouse chromosome 4 via the local hopping characteristic of SB transposons. To further characterize the effect of mobile transposons on genomic instability, we have used array comparative genomic hybridization (array CGH) to characterize copy number changes in tumors generated in the original SB insertional mutagenesis experiments (Collier and
Profiles of these tumors are shown in Figure 5. Surprisingly, we observed that tumors with mobile T2/Onc transposons displayed no copy number abnormalities, suggesting that transposon insertional mutagenesis is the primary route of mutation in the development of these tumors. Additionally, we observed copy number abnormalities in tumors with no mobile transposons that correspond to chromosomes harboring transposon insertions in tumors with mobile transposons (for example, the Braf gene on chromosome 6 was the most common T2/Onc transposon insertion site in the SB+ tumors, while SB- tumors gained an extra copy of chromosome 6). Together, this suggests that T2/Onc is a powerful mutagen that identifies genes on chromosomes relevant to “natural” tumor development (ie, chromosomes displaying copy number abnormalities), and that the local hopping of T2/Onc may not be necessary to identify genes on chromosome 4. This result also supports our proposed change in priority discussed in Task 1A, since T2/Onc integrations are
likely to uncover relevant tumor suppressors on chromosome 4, frequently deleted in mouse models of breast cancer.

Finally, efforts with relevance to our main project, and supported through DOD award have resulted in the development of an informatics tool, ProTIS, which uses the DNA structural characteristics of “preferred” Sleeping Beauty integration sites to identify likely transposon integration sites in genomic sequences (Geurts and Hackett, et al, 2006, Hackett, et al 2007). This tool can be used to determine whether transposon insertions in tumors are due to a bias for the sequence by the transposase, or due to oncogenic selection. This tool provides an additional filter to separate transposon insertions relevant to tumor development from background insertions due to preferences of the SB transposase.

**Task 3:** Identification of candidate genes using insertional mutagenesis (months 15-36)
A: Continue to monitor mice from task 2 (months 15-36): We are, in parallel, generating cohorts of MMTV-HER2 mice in which T2-Onc transposons are mobilized by CAGGS-SB10, RosaSB11, and Rosa-IsI-SB11. We are monitoring all of these mice for the onset of tumors.

**KEY RESEARCH ACCOMPLISHMENTS:**
- Sequence verification of 6 MICER targeting clones
- Cloning of V5-tagged 3’HPRT targeting vector
- Generation of a cohort of MMTV-HER2;CAGGS-SB10;T2-Onc mice
- Acquisition of alternative SB-expressing lines RosaSB11 and Rosa-IsI-SB11 and crosses into the MMTV-HER2 model system
- Cloning of retroviral and lentiviral vectors expressing SB11 transposase.

**REPORTABLE OUTCOMES:**
*Publications since award initiation:*

*Abstracts:*
2. Transposon-based somatic mutagenesis for cancer gene discovery. Lara S. Collier, David J. Adams, Laura E. Green, Eric P. Rahrmann, Michael N. Davies, Miechaleen D. Diers, Anthony J. Cox, Christopher S. Hackett, J. Graeme Hodgson, Adam J. Dupuy, Neal G. Copeland, Nancy A. Jenkins,
CONCLUSION:
To date, we have acquired and characterized all of the reagents necessary for this project. We have identified two reagents which do not function as expected in the context of mouse models of breast cancer (the 3’HPRT-GFP vector and the CAGGS-SB10 transposase construct). We have thus pursued alternative approaches. We have substituted an epitope-tagged 3’HPRT genomic targeting vector, and are in the final stages of validation for this construct. We have also acquired a wide range of tools to express the SB11 transposase in breast tissue; two additional mouse constructs, and 3 novel viral constructs. In parallel, we are re-confirming the genomic location of the targeting constructs we intend to use.

While the genomic targeting of our first aim has not progresses as rapidly as we expected, the Sleeping Beauty insertional mutagenesis system has progressed more rapidly than expected. We now have 3 mouse constructs with different temporal patterns of SB expression in breast tissue, allowing us to identify oncogenes and tumor suppressors via transposon insertion at different time points in tumor development. We have also developed viral vectors to deliver SB in vitro and control the exact time of activation; by transplanting virally-transduced tumor cells, we can identify changes late in tumor development. Additionally, these vectors may allow for in vitro screens for mutations involved in processes such as therapeutic resistance and relapse. Although our efforts over the first year are not yet capable of focusing T2/Onc insertions to the 1p36 region, insertion sites mapped in the preliminary cohorts of animals should have relevance to breast tumor development in general.

REFERENCES:


APPENDICES:
None.

SUPPORTING DATA:
Embedded in text.