

AD _____

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
San Francisco, CA 94143

REPORT DATE: April 2008

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.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 01-04-2008		2. REPORT TYPE Annual Summary		3. DATES COVERED 1 Apr 2007 – 31 Mar 2008	
4. TITLE AND SUBTITLE Characterizing the Role of 1p36 Deletion in Breast Cancer and Identifying Candidate Tumor Suppressors				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-06-1-0458	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Christopher S Hackett E-Mail: chris.hackett@ucsf.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California, San Francisco San Francisco, CA 94143				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 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 modified targeting constructs, generated cohorts of mice for insertional mutagenesis, identified transposon insertion sites, and developed in vitro, transplant-based alternative approaches.					
15. SUBJECT TERMS 1p36, insertional mutagenesis, sleeping beauty, mouse models, tumor suppressors					
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	10	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	9
Reportable Outcomes.....	9
Conclusion.....	10
References.....	10
Appendices.....	10

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. Patients whose tumors carry 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 identification 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. We have isolated breast tumors carrying the Sleeping Beauty insertional mutagen, and have successfully mapped genomic insertion sites for the mutagenic transposons.

BODY: Results from all 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:

As stated in last year's progress report, another group has published a study in which they constitutively deleted a 4 Mb region of mouse chromosome 4 within the 1p36 syntenic region (Bagchi, et al, 2007). While this study led to the identification of a candidate tumor suppressor (CHD5) in the region, mice harboring this deletion did not develop breast tumors (though they did develop a range of other tumors). Although careful reading of this study suggests that the biology of 1p36 deletion may be complex, requiring the deletion of multiple genes, a cursory reader might agree with the authors' contention that they have identified "the tumor suppressor" on 1p36. In light of this publication and taking the advice of my PhD thesis committee, I have de-prioritized the generation of the 1p deletion mouse, focusing primarily on the Sleeping Beauty insertional mutagenesis aspect of the project. Also,

New 3'HPRT targeting construct

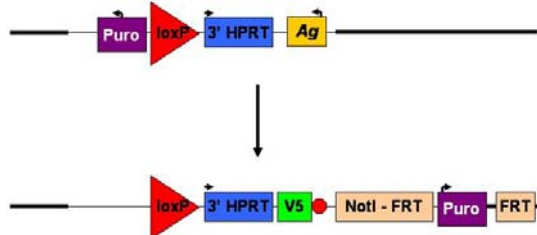


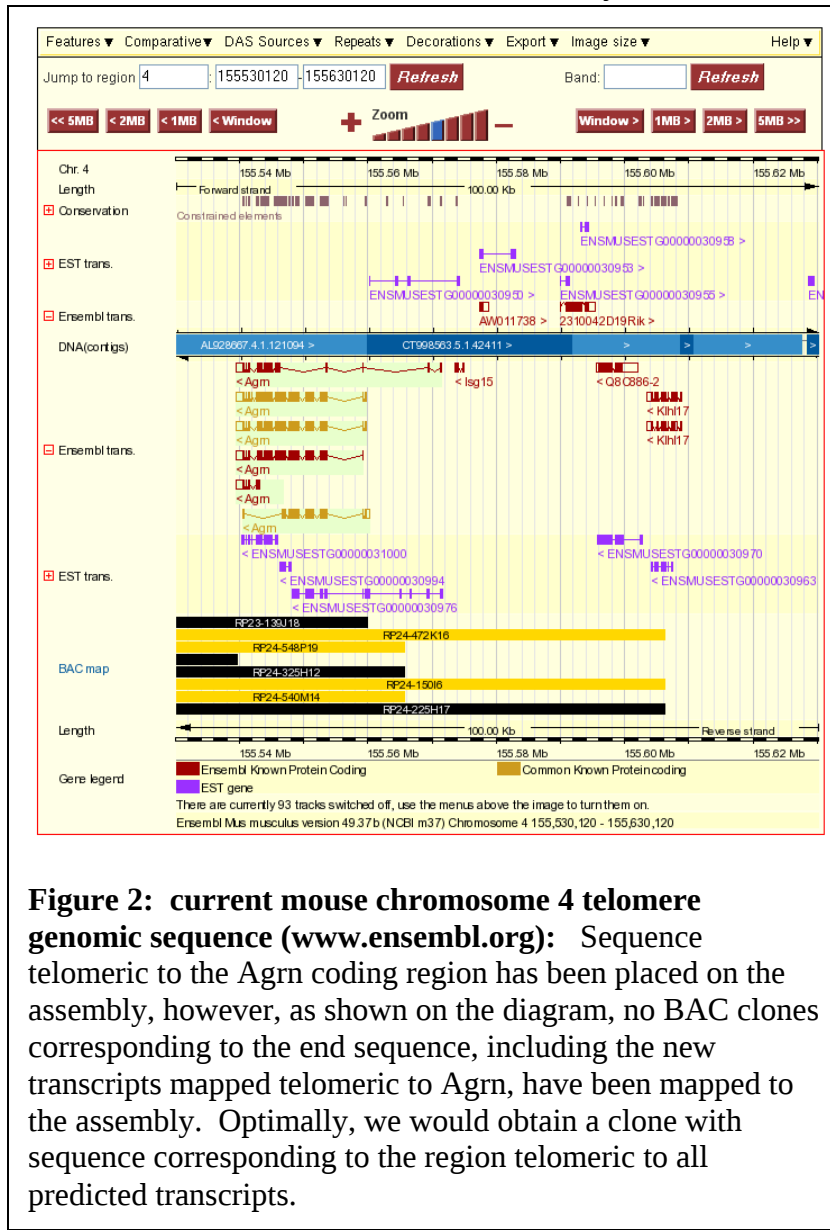
Figure 1: Modified 3'HPRT targeting vector: Vector has been re-engineered so as not to disrupt neighboring host genes in the opposite (right-to-left) orientation.

as stated in last year's report, 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. However, the genomic sequence assembly near the telomere has been altered twice since initiating this project. Initially, sequence was added such that the essential gene *Agrn* was included at the chromosome 4 telomere, with no genomic sequence beyond the *Agrn* cDNA alignment. In order to generate a construct that would not interfere with *Agrn*'s function, but would include *Agrn* in the induced somatic deletion, we have further re-engineered the 3'HPRT targeting contstruct (**Figure 1**). First, we removed the Agouti coat-color marker. We have also re-oriented the Puromycin cassette to lie downstream of the V5 epitope-tagged

3'HPRT coding region, in the same orientation. In ES cells, this construct will be flanked by FRT sites for subsequent removal after colony selection. This construct will neither activate nor trap any endogenous genes in the reverse orientation, and is suitable to be knocked into a large intron of the *Agrn* gene.

Since engineering initiating the alteration of the construct, however, the sequenced assembly changed again (Ensembl release December 2007). Currently, three more predicted genes have been mapped telomeric to *Agrn* (Figure 2). Unfortunately, no BAC clones are currently mapped to the assembly that contain the sequence downstream of these new transcripts, hindering our ability to FISH map the sequence or amplify flanking genomic sequence for the targeting constructs. We hope that BAC clones will be mapped to this sequence by the time our targeting construct is finished.

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, we have not performed the ES cell targeting to date.

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. We have generated MMTV-Cre;MMTV-HER2 mice and are using them in Task 3.

Task 2: Characterization of 1p36 deletion in the context of Her2-initiated oncogenesis: Since initiation of Task 2 is dependent on Task 1, it has not yet been initiated.

Task 3: Identification of candidate genes using insertional mutagenesis (months 15-36)

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.

mouse	type	barcode	Sequences
8354 T1	breast	GTGATTACAC	119
8354 T2	breast	ATAAGATAAC	75
8354 T3	breast	CTGTAGGATC	164
8355 T1	breast	GCTACATGAT	409
8355 T2	breast	TATAGACGCC	151
8355 T3	breast	TGCAGATCGA	75
9295 T1	breast	ACGCTAGTGT	221
9295 T2	breast	AGCCGCCGAT	213
8927T1	breast	TCTGGCTTGA	124
9439T2	breast	ATAACTCGCG	74
9439T3	breast	TAATAGATTG	266
total			1891

Table 1: pyrophosphate sequence reads for 11 HER2 tumors

To take advantage of this system, we have generated a cohort of triply-transgenic MMTV-HER2;CAGGS-SB10;T2-Onc mice to test the mobilization of SB transposons in breast tumors. We have isolated over 40 mammary tumors from triply-transgenic mice. We have utilized 454 pyrophosphate sequencing to identify transposon insertion sites from 11 tumors from 5 of these animals.

Using this system, each tumor is barcoded, such that the resulting sequence reads can be matched to a specific sample. The number of sequences returned per sample is shown in Table 1.

We also sequenced insertion sites in DNA from spleens from tumor-prone animals. We use spleen

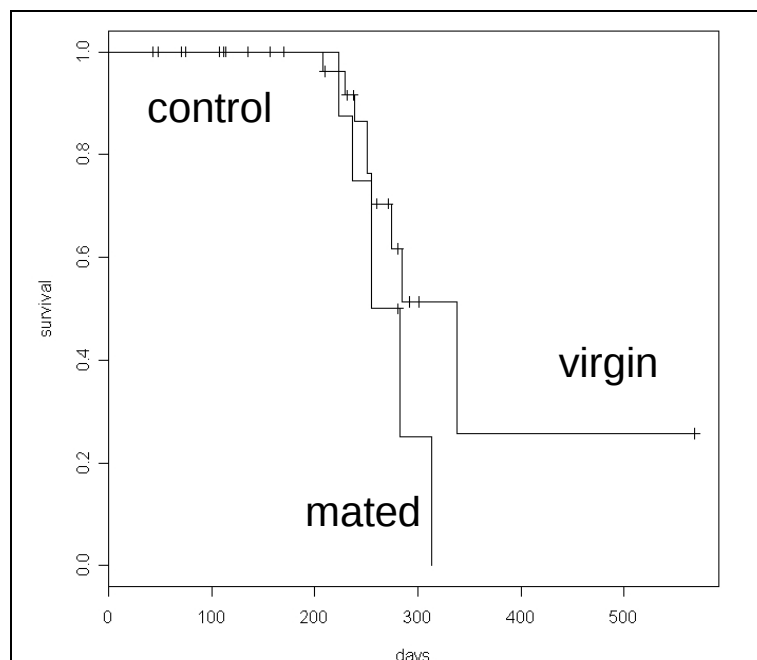


Figure 3: Survival of MMTV-HER2/neu;CAGGS-SB10;T2-Onc females: 40 virgin females and 9 mated females shown. A control cohort of 10 MMTV-HER2 virgin females is currently being aged. While mating accelerates tumor onset, median time to onset is not notably different from traditional controls. Our cohort will be compared with an in-house cohort to assess whether they show a statistically-significant decrease in time to onset.

sequences to distinguish insertions during development from insertions arising in tumors. Importantly, several mice developed multiple tumors. In many cases, the pattern of transposon insertions was similar in distinct tumors, and in some cases, similar to transposon insertions mapped in spleen. We speculate that since transposase was active throughout development in many tissues, these insertions represent systemic insertions that did not arise during tumor development. Nevertheless, and in spite of the fact that tumors from only 5 animals were sequenced, our preliminary analysis suggests four genomic loci carrying insertions in multiple tumors. Verification of this data is ongoing, but suggest that identification of CISs in this system is feasible. We have isolated 40 tumors from these animals, and are mapping insertion sites in these tumors (and spleen controls) using 454 pyrophosphate sequencing. 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 in adult animals. 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 (not shown; discussed in last-year's status report). However, preliminary IHC analysis suggested that the construct may be active in advanced tumors. We have now plotted survival curves to determine whether the Sleeping Beauty system is accelerating tumorigenesis in these animals (Figure 3). However, a significantly-sized control cohort has not been tracked long enough to determine the baseline average tumor onset; this control will reach the necessary age window within the next year.

While we will continue to monitor the cohort of mice with the CAGGS-SB10 construct, we have in parallel pursued 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 have generated a cohort of Rosa-lsl-SB11;T2-Onc2(high-copy) mice carrying either MMTV-Cre alone or MMTV-HER2;MMTV-Cre. These mice should activate SB transposase in breast tissue exclusively. A summary of the current cohort sizes for these groups is presented in Table 2.

		mice alive	controls	tumors
MMTV-Cre	MMTV-Cre/RSBLSL/T2-onc2	53	27	1
MMTV- HER2/neu	MMTV-HER2/SB10/T2-onc	28	10	40
	MMTV-HER2/RSB11/T2-onc	4	21	0
	MMTV-HER2/RSB11/T2-onc3	0	24	0
	MMTV-HER2/MMTV-Cre/RSBLSL/T2-onc2	14	4	0

Table 2: Cohort sizes for MMTV-HER2-based sleeping beauty studies

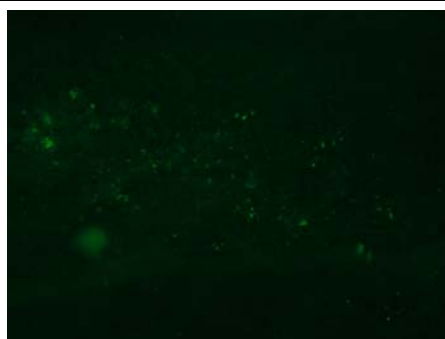


Figure 4: Growth of GFP-positive transplanted tumor cells in mammary fat pads:

Cells harvested from MMTV-HER2;T2/Onc tumors were isolated and transduced with pMIG or pMIG-SB11 in vitro, then re-transplanted into recipient mammary fat pads. Transplanted cells failed to develop into GFP-positive tumor masses.

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-IRES-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 (discussed in last year's status report).

We have isolated tumor cells from tumors generated in MMTV-HER2;T2-Onc mice (with "dormant" transposons) and transduced pMIG-SB11 constructs in-vitro. We have attempted to transplant these cells into recipient FVB/MTV-HER2 mice (**Figure 4**). However, none of the mice injected developed GFP-positive tumor masses. Collaborators have also had difficulty transplanting cells derived from MMTV-HER2 tumors. We are currently exploring alternatives, including transplantation of cells carrying the oncogenic construct MMTV-c-myc and T2/Onc, as well as normal mammary epithelial cells

carrying various transposons. In parallel, we are generating a cohort of mice carrying the MMTV-c-myc construct similar to the groups described in Table 3 for MMTV-HER2.

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-lsl-SB11. We are monitoring all of these mice for the onset of tumors.

KEY RESEARCH ACCOMPLISHMENTS:

- Generation of a cohort of MMTV-HER2;CAGGS-SB10;T2-Onc mice
- Isolation of tumors, mapping of transposon insertion sites, identification of common insertion sites
- Acquisition of alternative SB-expressing lines RosaSB11 and Rosa-lsl-SB11 and crosses into the MMTV-HER2 model system

REPORTABLE OUTCOMES:

Publications since award initiation:

1. Hackett, CS, Geurts, AM, Hackett, PB. **Predicting preferential DNA vector insertion sites: implications for functional genomics and gene therapy.** Genome Biology (in press).
2. Cheng AJ, Ching Cheng N, Ford J, Smith J, Murray JE, Flemming C, Lastowska M, Jackson MS, Hackett CS, Weiss WA, Marshall GM, Kees UR, Norris MD, Haber M. **Cell lines from MYCN transgenic murine tumours reflect the molecular and biological characteristics of human neuroblastoma.** Eur J Cancer. 2007 Apr 19 [Epub ahead of print]
3. Geurts AM, Hackett CS, Bell JB, Bergemann TL, Collier LS, Carlson CM, Largaespada DA, Hackett PB. **Structure-based prediction of insertion-site preferences of transposons into chromosomes.** Nucleic Acids Res. 2006 May 22;34(9):2803-11.

Abstracts:

1. **Strain-specific penetrance and chromosome copy number variations in a mouse model for neuroblastoma.** Christopher S. Hackett, J. Graeme Hodgson, Jian-Hua Mao, Denise Lind, Natalie Blades, Gary Churchill, Javed Khan, Pui-Yan Kwok, Allan Balmain, and William A. Weiss. AACR Mouse Models of Cancer Conference October 25-28, 2006.
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, William A. Weiss, Allan Bradley, Paul C. Marker and David A. Largaespada AACR Mouse Models of Cancer Conference October 25-28, 2006.
3. **Structure-Based Prediction of Insertion-Site Preferences into Chromosomes of Vectors used for Gene Therapy.** P.B. Hackett, C.S. Hackett, and A.M. Geurts American Society for Gene Therapy, 2007 (Submitted)

Awards:

AACR Scholar-in-Training Travel Award (AstraZeneca), AACR Mouse Models of Cancer Conference October 25-28, 2006.

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 re-engineered the 3'HPRT genomic targeting vector for a new genomic targeting site, 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. We have generated a significant cohort of mice and have isolated over 40 tumors. We have sequenced insertion sites from 11 of these tumors, and have seen indications of common insertion sites.

While the genomic targeting of our first aim has not progressed as rapidly as we anticipated, 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 two years 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:

Bagchi A, Papazoglu C, Wu Y, Capurso D, Brodt M, Francis D, Bredel M, Vogel H, Mills AA. CHD5 is a tumor suppressor at human 1p36. Cell. 2007 Feb 9;128(3):459-75.

APPENDICES:

None.

SUPPORTING DATA:

Embedded in text.