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AWARD NUMBER: DAMD17-02-1-0345

TITLE: Searching the Epigenome for Novel Breast Cancer Tumor Suppressors

PRINCIPAL INVESTIGATOR: Greg J. Hannon, Ph.D.

CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

REPORT DATE: September 2005

TYPE OF REPORT: Final

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-09-2005	Final	1 Sep 2002 – 31 Aug 2005

4. TITLE AND SUBTITLE

Searching the Epigenome for Novel Breast Cancer Tumor Suppressors

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0345

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Greg J. Hannon, Ph.D.

E-Mail: hannon@cshl.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

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

Our initial proposal focused on developing technologies to uncover epigenetic changes that contribute to tumor development. Our initial attempts towards developing genome wide approaches to identify new genes silenced by epigenetic mechanisms encountered problems; however, our efforts to exploit epigenetic mechanisms of gene silencing to study tumor suppressor gene function have been very successful (see below). We have built upon these successes both to push the development of broadly useful technologies for the use of RNAi in cell culture, in transgenic animals and in mosaic animals. This has led to insights into the function of tumor suppressor genes and is leading toward the identification of epigenetic regulators that are potentially cancer relevant. Finally, we have found that senescent cells for heterochromatic foci, SAHFs, which may help this innate tumor suppression mechanism to maintain stable growth arrest.

15. SUBJECT TERMS
Cancer Biology, Genetics, Synthetic Lethality, Apoptosis

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	8	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Final Report

Introduction

Our work under this application continues to be geared toward understanding how changes in epigenetic regulation contribute to tumor development. We have sought to understand epigenetic changes as they relate to tumorigenesis from several perspectives. These are described below.

Body/
Key Accomplishments

1. Control of Cellular Senescence. Cellular senescence is an extremely stable form of cell cycle arrest that limits the proliferation of damaged cells, including cells encountering telomere malfunction or DNA damage. As a consequence, mutations that disable senescence contribute to cellular immortalization and drug resistance in breast epithelial cells and other cell types. Work from our groups and others indicate that the p53 and p16/Rb tumor suppressor pathways are crucial regulators of senescence, but how activation of these pathways leads to a permanent arrest has remained largely unexplored. This year, we described a distinct heterochromatic structure that accumulates in senescent human fibroblasts, which we designated senescence-associated heterochromatic foci (SAHF) (Narita et al., 2003; Narita and Lowe, 2004). SAHF formation coincides with the recruitment of heterochromatin proteins and the Rb tumor suppressor to E2F-responsive promoters, and is associated with the stable repression of E2F target genes. Notably, both SAHF formation and the silencing of E2F target genes depend on the integrity of the Rb pathway, and do not occur in reversibly arrested cells. These results provide the first insights into the effector mechanisms of cellular senescence, and indicate that the process is under epigenetic control. Since we believe that senescence is a potent mechanism of tumor suppression, we are continuing to characterize this program with the goal of identifying new players that may contribute to breast and other cancers.

2. Analysis of the CBX7 oncogene. We are collaborating with David Beach to characterize the in vivo properties of the putative oncogene, CBX7. CBX7 is a member of the polycomb group family that was identified by virtue of its ability to immortalize epithelial cells in culture (Gil et al., 2003). Previous work indicates that Bmi-1, another polycomb group protein that can form a complex with CBX7, is oncogenic when overexpressed in mice. Moreover, disruption of Bmi-1 leads to stem cell depletion, suggesting Bmi-1 can contribute to stem cell maintenance. To determine whether CBX7 has similar properties, we produced chimeric mice that expressed CBX7 in the hematopoietic compartment. We showed that CBX7 is a potent oncogene in vivo, capable of both initiating tumorigenesis and cooperating with c-myc to accelerate the onset of malignancies (Scott et al, in preparation). We are currently developing short hairpin RNAs (shRNAs) to suppress CBX7 function, and intend to use them to determine whether CBX7 acts as an oncogene by controlling the INK4a/ARF locus and/or influences stem cell maintenance. Since p16INK4a is an important tumor suppressor in breast cancer, we hope these studies will help elucidate how epigenetic control of its expression can influence normal cell function and cancer development.

3. Use of RNAi to dissect tumor phenotypes. The application of RNA interference (RNAi) to mammalian systems has the potential to revolutionize genetics and produce novel therapies. With support from this grant, we investigated whether RNAi could be used to

suppress gene expression in vivo and produce phenotypes in mice. We showed that shRNAs directed against Neil1, a gene involved in DNA repair, could suppress gene expression in transgenic animals leading to a radiosensitive phenotype (Carmell et al., 2003). Furthermore, we used a murine lymphoma model to show that shRNAs against the p53 tumor suppressor could accelerate myc induced lymphomagenesis (Hemann et al., 2003). Interestingly, different *p53* shRNAs produced distinct phenotypes *in vivo*, ranging from benign lymphoid hyperplasias to highly disseminated lymphomas that paralleled the nullizygous setting. In all cases, the severity and type of disease correlated with the extent to which specific shRNAs inhibited p53 activity. Together, our results show that RNAi can stably suppress gene expression in stem cells and reconstituted organs derived from those cells. Moreover, intrinsic differences between individual shRNA expression vectors targeting the same gene can be used to create an 'epi-allelic series' for dissecting gene function *in vivo*.

Based on the above results, we are now applying RNAi technology to study other known or putative tumor suppressor genes in vivo. For example, we examined the possible tumor suppressor properties Bim and Puma, two "BH3-only" pro-apoptotic members of the Bcl-2 family. Bim contributes to apoptosis in several settings, including anoikis and taxol-induced apoptosis in breast epithelial cells and carcinomas, respectively (Renato et al., 2003; Sunters et al, 2003). Puma is a direct p53 target that appears to be an important p53 effector in drug induced apoptosis (Villunger, et al., 2003; Jeffers et al., 2004). Using the shRNA technology and rapid transgenic technologies we have developed, we showed that disruption of either Bim or Puma cooperate with myc during tumorigenesis (Hemann, et al., 2004). Moreover, we are currently testing whether suppression of Puma or Bim can promote drug resistance (preliminary studies indicate yes) and whether this is agent dependent.

4. RNAi libraries and other new tools. Over the term of this award, we also have made advances in RNAi expression technology. For example, with funds from a variety of sources, we have developed comprehensive shRNA libraries covering the majority of genes in the mouse and human genomes. These libraries comprise more than 150,000 arrayed, sequence-verified shRNA constructs in flexible expression vectors. These are presently available to the scientific community as a whole and are being applied to support the goals of this idea award.

We also have developed conditional strategies for shRNA expression. These have been described in two recent publications (Dickins et al. 2006, Stegmeier et al, 2006) which show that conventional conditional expression strategies can be combined with our second-generation shRNAs for regulated suppression of gene expression either in vitro or in vivo. Importantly, we are now applying these strategies in the context of both transgenic animals and mosaic models of breast cancer that rely on mammary gland reconstitution with engineered stem cells.

5. Using RNAi libraries to understand mechanisms of stable epigenetic control

Stable changes in the state of gene expression are key to tumor development. Specifically, silencing of tumor suppressor alleles by DNA and histone methylation occurs frequently. Thus, understanding mechanisms of stable epigenetic control is critical for illuminating how errors in this machinery contribute to tumor development.

One of the most fascinating examples of sequence-specific gene regulation is the phenomenon of genomic imprinting. Briefly, genomic imprinting is the process where a single allele of a given gene is silenced based on inheritance from either the mother or father. This

process is of great relevance to proper embryonic development and loss of imprinting is also associated with a large number of aggressive cancers. We are employing an RNAi based genetic screen to identify candidate genes that are involved in maintaining imprinted gene expression in a cell culture system.

This screen makes use of a cell line in which the Neomycin antibiotic resistance gene has been knocked into the Zac1 locus which contains an imprinted candidate tumor suppressor gene. The Neomycin resistance gene is active when inherited maternally but inactive when paternally derived. Using a library of small hairpin RNAs (shRNAs) targeting a list of approximately 150 genes known to be involved in epigenetic gene regulation we have silenced each of these genes and screened for those cells that lose the capacity to maintain the epigenetic silencing of the Neomycin resistance gene and thus become G418 resistant. Expression of Neomycin from this imprinted locus is tightly suppressed but can be reactivated in culture upon a high dose of the demtethylating agent 5-aza-cytidine. Suppression of individual candidate genes via RNAi offers a high throughput method to screen for epigenetic repressors of this locus and the imprinted Neomycin gene offers a method for positive selection.

Initial work on this project focused on finding an appropriate vector backbone from which to express the shRNAs. In an effort to improve our existing plasmid based shRNA expression system we sought to develop an shRNA expression cassette whose product would mimic an endogenous microRNA. It is currently hypothesized that shRNAs are processed in similar fashion to endogenous microRNAs and thus any modification to our shRNA expression system that improves processing should result in increased silencing ability. To this end we designed new shRNAs that are flanked by sequences of the mir30 miRNA and demonstrated that this design strategy improves shRNA processing. Further we have tested the ability of different classes and types of promoters to dive the expression of our mir30 context shRNAs and find that Poll II promoters work best in our assays.

Upon development of an optimal shRNA expression vector, the shRNA expression cassette was cloned into a Mouse Stem Cell Virus backbone (MSCV). Using an MSCV based vector will facilitate genomic integration and thus the stable expression of the shRNA. The Epigenetic shRNA Library was cloned as a pool into the MSCV – Mir shRNA vector and used to generate recombinant virus carrying the shRNA transgenes. Virus was then used to infect the Zac-Neomycin cells described above in single copy i.e. one unique shRNA from the library targeting one of the 150 candidate genes per cell. These cells were grown for five days before being subjected to one of two experimental paradigms.

In the first experimental model we sought to look for genes whose suppression would completely relieve the silencing of the Zac1 locus and thus relieve suppression of the Neomycin gene conferring stable G418 resistance to the cells. Cells were grown in media containing G418 for a period of 14 days after which resistant colonies were picked and expanded. Genomic DNA was isolated from the colonies and subject to PCR analysis to determine which shRNA was present in the genome. From this screen we identified Dnmt3a as well as HDAC2 and Myst1. Dnmt3a is a DNA methyltransferase known to be involved in establishing the DNA methylation mark of several imprinted genes and thus its identification as a potential candidate gene is not surprising. However, neither HDAC2 nor Myst1 have been identified as having any specific involvement in imprinting.

In the second experimental model we wanted to search for candidate genes that would confer a partial resistance to G418 i.e. a partial relief of Neomycin suppression. This model is similar to studies which look for resistance to a cell death phenotype in that knock down of the candidate gene may sufficient to intermittently permit transcription of the Neomycin resistance gene but is unable to completely relieve its suppression. Cells were grown for 5 days in G418 containing media after which the media was removed, cells washed and allowed to recover for 9 days in regular growth media. At the end of this period, colonies were again picked and genomic DNA isolated. Sequencing of the shRNAs present in these colonies revealed 12 candidate genes, most originating from the NURD chromatin remodelling complex. In fact, six of the 12 candidate genes identified in this screen are known components of the NURD complex. HDAC2 a chromatin modifying enzyme and central component of the NURD complex was again identified. In addition to the NURD complex, two components of the Swi/Snf chromatin remodelling complex were identified. Swi/Snf1 and SwiSnf5, emerged with Swi/Snf1 being identified via the suppressive actions of four separate shRNAs in multiple independent colonies. Clearly the suppressive actions of two key chromatin remodelling complexes are involved in the suppression of the imprinted Zac1 locus.

Of interest, two genes of unknown function were also identified in these two screens. Of all the candidates to emerge, MYST1 and MYST4 are perhaps the most interesting. Both were identified by multiple shRNAs in independent colonies with MYST1 being able to confer G418 resistance. The Drosophila homologues of these two genes have a putative role in dosage compensation but their biochemical function in mammalian cells is not understood at all. We are currently in the process of verifying each of these candidate genes.

In recent years, epigenetics has been recognized as a major factor in the development and progression of cancer. Epigenetic inactivation of genes is as major a factor leading to gene silencing during tumor progression, as is genetic mutation. As such, it has been included in the Knudson's two-hit hypothesis of cancer development. Studies of the mechanisms and biochemistry at work herein have recently moved to the forefront of cancer research and it is now known that epigenetic silencing of tumor suppressor genes is a hallmark of cancer progression. Further, studies indicate that as cancer development proceeds, tumors are able to epigenetically modulate their specific gene expression profiles in response to tumor microenvironments and then pass these changes on to progenitor cells. With each gene silenced or reactivated, the tumor cells gain a selective growth advantage. Epigenetic silencing of specific genes involved in maintaining cell cycle control or chromosomal stability further propel tumor cells into an aggressive state of proliferation and make clinical treatment difficult.

The Zac1 locus carries the imprinted Zac gene which is a well characterized candidate tumor suppressor gene involved in several differentiation processes and its expression is lost in a host of cellular carcinomas. In addition to identifying several of the key factors involved in maintaining imprinted gene silencing, this screen also has the potential to identify therapeutic targets for those cancers in which Zac expression is lost through an epigenetic based silencing mechanism.

Reportable outcomes

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Conclusions

Over the period of this grant, we have made progress on several levels toward understanding mechanisms of epigenetic control that are relevant to tumorigenesis. These relate to cellular senescence, a key element of endogenous tumor suppression programs, and more recently to mechanisms of epigenetic control at an imprinted locus. We have also used the support from this award to develop and distribute tools to the scientific community (for example inducible shRNA expression systems) that will have a broad impact in the breast cancer research community.