



FINAL REPORT

Project Title: Identification of aberrantly methylated genes in Ewing sarcoma

Project Number: SFA09-06

1. Date project was initiated: June 1, 2009
2. Period covered by this report: From June 1, 2009 To June 30, 2010
3. Publications, Abstracts, and Presentations:

- a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry must include the author(s), article title, journal [book, editor(s), publisher, volume number, page number(s), and date.]

We have decided to delay submission of our manuscript until we can better characterize the role methylated genes identified by our screen. We anticipate manuscript submission in the Fall 2010 or Winter 2011.

- b. List presentations made during the last year (international, national, local societies, etc.). Use an asterisk (*) if presentation produced a manuscript.

**Borinstein S.C., Patel N.J, Marcondes A.M., and Grady W. M.
CALCA, SERPINB5, and NNAT are Epigenetically Silenced by DNA
Hypermethylation in Ewing Sarcoma Primary Tumors and Cell Lines. Poster
Presentation, Vanderbilt University Department of Pediatrics Research Day, June 12,
2010, Nashville, TN. Poster # 009**

4. Provide a brief list of keywords: (limit to 20 words)

Epigenetics, DNA Methylation, Sarcoma, Ewing's Sarcoma, Pediatrics

5. Summarize the progress during the period of this report and its impact on your plans for the remainder of the project. Include a summary of the progress toward the achievement of the originally stated aims and list the significant results:

Acquisition of ES tumor samples, clinicopathologic data, and methylation analysis

The primary goal of the proposal was to identify aberrantly methylated genes in Ewing Sarcoma (ES). As of September 1, 2010, I moved from the Fred Hutchinson Cancer Research Center to Vanderbilt University Medical Center (VUMC). Upon arriving at Vanderbilt, we fostered collaboration with Cheryl Coffin and Jennifer Black, pathologists at VUMC who have been extremely helpful in the acquisition of primary ES tumor samples. After receiving approval from our IRB, we gained access to the pathology archives and medical records of patients with ES treated at Vanderbilt. We identified over 110 patients diagnosed with ES between 1990-2009 between the ages of 1 and 50 years. All patients had complete electronic medical records that allowed for collection of clinicopathologic information (including age at diagnosis, location of primary tumor, tumor stage, and evidence of EWS-Fli1 translocation). We recorded these data along with treatment response and survival outcome. We were also able to identify the formalin-fixed paraffin embedded tissue blocks that corresponded to each patient stored in the VUMC archives. The blocks were sectioned, verified to be ES by pathologist review, and DNA was extracted using RecoverAll™ (Applied Biosystems) and quantitated using PicoGreen fluorimetry (Invitrogen). We were able to isolate sufficient quantities of DNA from 58 FFPE tissue blocks. At least 100 ng of genomic DNA for each samples was then used for methylation analysis. Methylation analysis was then performed using the Goldengate technology with the Methylation Cancer Panel I beadchip (Illumina), which simultaneously interrogates the methylation status of over 1500 CpG dinucleotides located in 800 genes known to be altered in cancer in 96 samples in parallel. We compared the methylation profiles of 58 primary ES tumors to human bone marrow stroma cell cultures, which are an adequate representation of human mesenchymal stem cells (hMSC), thought to be the cell of origin for ES.

Identification of genes Aberrantly Methylated in ES

We had previously analyzed 13 primary tumors, 3 ES cell lines, and 4 hMSC cultures using Goldengate Methylation analysis. We combined the results of the previous experiment with our new data set, resulting in the analysis of methylation from 71 primary ES tumors and 8 hMSC samples. The data was filtered to only include CpGs dinucleotides that were located in CpG islands in the promoter region of known genes (defined as being -700 to +300 of the transcription start site). We also excluded CpGs located on the X chromosome due to genetic imprinting. Using p-value and β scores as defined by Illumina's GenomeStudio software, we identified genes that were methylated in >30% of ES primary tumors and unmethylated in at least 80% of hMSC samples, which resulted in the identification of 53 CpGs in 51, shown in Figure 1. Genes involved in cell cycle control, apoptosis regulation, tumor invasion, development, and tumor suppression are included on this list.

One of the genes included on this list is *CALCA*, which encodes the calcitonin propeptide. Calcitonin is a hormone involved in calcium homeostasis which has also been demonstrated to be hypermethylated in bladder, thyroid and cervical cancer. Furthermore, *CALCA* is often methylated in pediatric MDS and ALL. We validated the methylation results for *CALCA* using Methylation Specific PCR (MSP) and bisulfite sequencing (figure 2). Furthermore, treatment of ES cell lines with the demethylating agent 5-aza-2'-deoxycytidine resulted in an increase in gene expression (figure 2), suggesting that *CALCA* is epigenetically silenced in ES. Our laboratory is currently investigating the role of *CALCA* in the pathogenesis of ES.

Correlation of clinicopathologic data with methylation status

The second aim of this project was to correlate treatment response with methylation. We are currently analyzing the methylation data set to determine if gene methylation status correlates with clinicopathologic features of the tumor such as age, sex, location of the tumor in addition to clinical outcome. So far we have found that several genes are methylated in a subset of ES tumors that may potentially correlate with overall survival. However, more extensive statistical analysis is required to better understand the implications of these preliminary results.

6. In layperson's terms, summarize the progress during the period of this report. Explain any medical significance or implications of your results to date:

The goal of this research project is to identify new ways to diagnose and treat Ewing Sarcoma. DNA methylation is a way that cancers can turn off genes that would normally prevent tumors from growing. Using new technology, we were able to search for genes that are methylated in Ewing Sarcoma, with hope of identifying new targets in the development of novel, effective treatments for patients suffering from this horrible disease.

By using powerful new analytical techniques, we have been able to successfully isolate DNA from pathology tissue blocks (some up to 20 years old!) from patients with Ewing Sarcoma. We were also able to identify a group of genes that are frequently methylated in these tumors when compared to normal cells. Currently we are trying to better understand how methylation contributes to Ewing Sarcoma tumor formation and spread, and to determine if methylation of certain genes could play a role in their diagnosis or treatment. The data we have generated, thanks to the SFA grant, has been a great start in the understanding of how DNA methylation plays a role in Ewing sarcoma biology.

Appendix: Figures 1 and 2

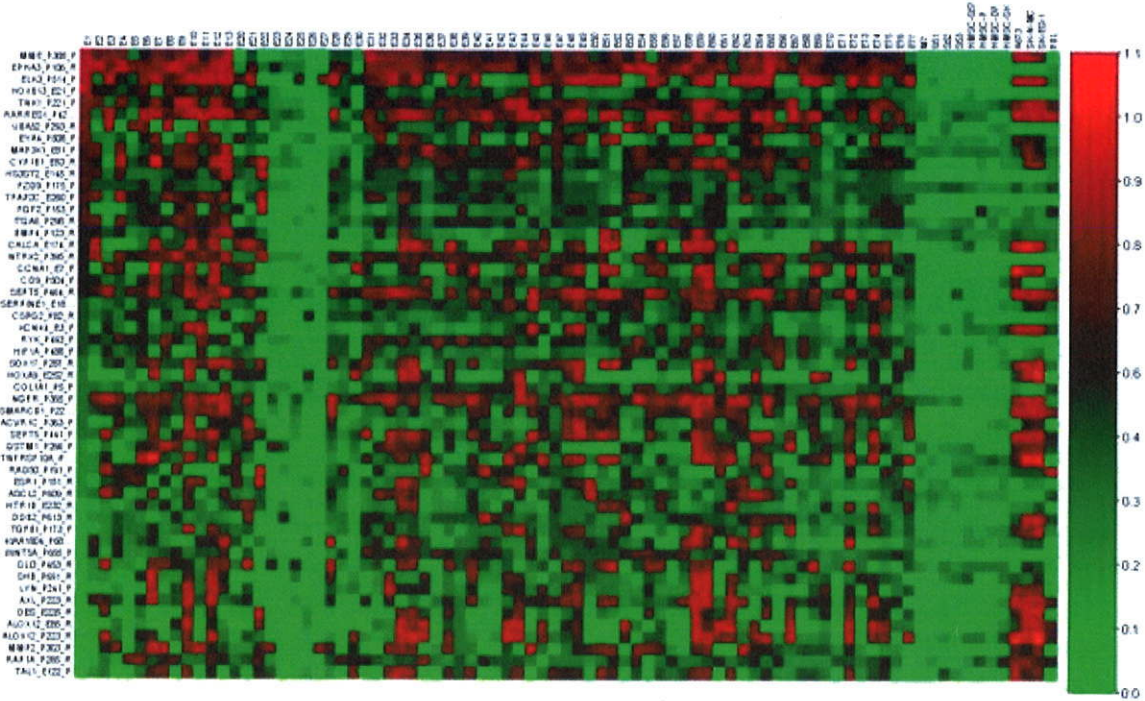


Figure 1: Methylation Bead Array Analysis. B) Heat map depicting genes hypermethylated in >30% of EWS primary tumors and cell lines when compared to human mesenchymal stem cells (hMSCs). Red blocks denote methylation and green blocks represent unmethylated genes. The primary EWS tumors are designated E1-E13 and E20-E77 and the human mesenchymal stem cell (hMSC) samples follow (M7, S51, S52, S53, hMSC-S57, hMSC-P, hMSC-DP-, and hMSC-SK). Also shown are the methylation signatures of the ES cell lines A673, SK-N-MC, and SK-ES-1 and peripheral blood leucocytes (PBL).

*Reports due by 31 July 2010

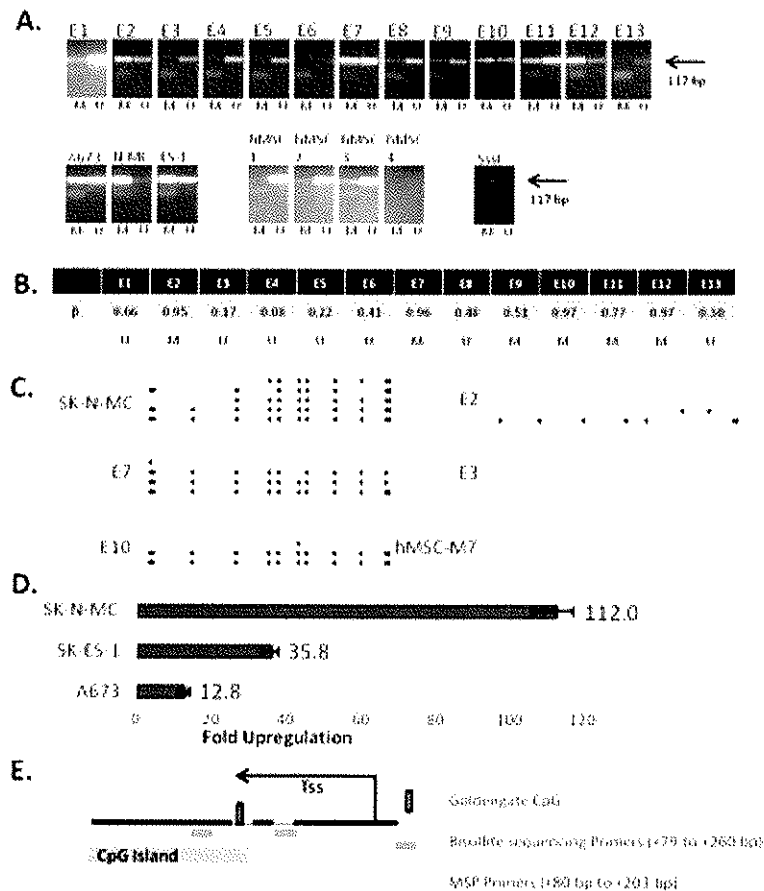


Figure 2. CALCA methylation and gene expression analysis. A) MSP analysis of ES primary tumors (E1-E13), ESS cell lines, and hMSCs. M = methylated; U = unmethylated. SssI treated DNA was used as a positive control. B) β -values from Goldengate analysis C) Bisulfite sequencing of CALCA in EWS cell lines, hMSCs, and EWS tumors E2, E3, and E10. Lollipop diagram depicts methylated CpGs (filled circle) or unmethylated CpG (empty circle) at each CpG dinucleotide in the analyzed region. D) Quantitative Real Time PCR (qRT-PCR) for CALCA. TaqMan probes were used to determine the gene expression of CALCA in EWS cell lines with or without 5-AZA treatment. Error bars demonstrating the standard error of the mean (SEM) are shown. E) Schematic diagram of CALCA showing the location of the TSS in relation to the CpG island, MSP primers, bisulfite sequencing primers, and the CpG analyzed by the Goldengate Assay



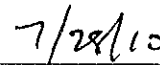
Principal Investigator (signature)



Date



Department Chair (signature)



Date