The Wistar Institute of Anatomy and Biology

Annual Progress Report: 2012 Formula Grant

Reporting Period


Formula Grant Overview

The Wistar Institute of Anatomy and Biology received $1,491,186 in formula funds for the grant award period January 1, 2013 through June 30, 2014. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Informatics Solutions for NextGen Sequence Data Analysis – Recent genome-wide studies suggest that at least half of the human genes, including many therapeutically target genes, produce multiple protein isoforms through alternative transcription. Many of the isoforms produced in this manner are tightly regulated during normal development but are mis-regulated in cancer cells. We propose to develop novel algorithms and build an informatics platform for understanding gene regulation at isoform-level (alternative promoter or alternative transcript-level) by developing statistically rigorous bioinformatics resources for processing Next-Generation Sequencing (NGS) data to better understand gene regulatory mechanisms in mammalian cells, and more importantly, how dis-regulation of these mechanisms leads to cancer. The informatics platform will be tested by analyzing The Cancer Genome Atlas data.

Anticipated Duration of Project

1/1/2013 – 6/30/2014

Project Overview

In recent years, the notion of “one gene makes one protein that functions in one signaling pathway” in mammalian cells has been shown to be overly simplistic. Recent evidence suggests that more than 50% of the human genes produce multiple protein isoforms, through alternative splicing and alternative usage of transcription initiation and/or termination. Notably, the disruption of many of these genes is implicated in cancer and several neuropsychiatric disorders. For majority of human genes the resulting multiple protein isoforms are functionally different and can participate in different signaling pathways. We propose to build an informatics platform for understanding gene regulation at isoform-level by developing statically rigorous bioinformatics resources for processing Next-Generation Sequencing (NGS) data. Recently, computational approaches that combine seemingly disparate experimental data have been successful in developing concise gene regulation models and transcriptional modules. We plan to extend these methodologies to perform integrative analysis of multiple high-throughput data sets,
such as The Cancer Genome Atlas (TCGA) datasets. We will apply innovative statistical modeling approaches that combine state-of-the-art meta-classification algorithms, such as Naïve Bayes Tree, Bagging and LogitBoost, with Random Forest feature selection to classify different types of target promoters with good classification accuracy and reduced instability, in order to predict differentially active gene promoters, their transcript variants and infer the protein-DNA interactions from RNA-seq and ChIP-seq data. This will be completed by pursuing the following aims, (1) Develop statistically rigorous novel algorithms and bioinformatics pipelines to identify the orthologous promoters, corresponding transcript variants and protein isoforms that are conserved between human and mouse, and (2) develop novel algorithms and informatics pipelines for integrative analysis of NGS datasets to estimate the activity and expression of both known and novel promoters and their transcript variants, in various tissues, developmental stages, and different cancer types by data-mining of The Cancer Genome Atlas data.

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Expected Research Outcomes and Benefits

An important question of immediate attention that will be addressed by this project is “which alternative promoters and their transcript variants of human genes are preferentially silenced or activated in cancer cells and different cancer types”. This project will develop novel bioinformatics methods to determine the orthologous human-mouse pairs of primary and alternative promoters, their corresponding transcript variants and the protein isoforms they encode. And, novel algorithms will be developed to estimate the activity of alternative promoters and alternative transcripts in different tissues and their developmental stages and cancer types, from integrative analysis of NGS datasets. We will test and validate various statistical machine-learning methods in the development of efficient bioinformatics pipelines. The broad potential impact on the field of biomedical informatics is development of novel algorithms and informatics pipelines for in silico discovery and research for accelerating the linkage of phenotypic and genomic information, at gene-isoform level by making use of NGS data. We believe this research will identify a large number of novel targets for the diagnosis and treatment of different cancers. We have earlier discovered the wide-spread use of alternative promoters and splicing in mammalian genes by using the ChiP-seq and RNA-seq approaches in brain development. For example, alternative splicing of VEGFA gene yields multiple proangiogenic, and paradoxically, anti-angiogenic isoforms. Accordingly, for the majority of glioblastoma "driver" genes, the inclusion/exclusion of exonic sequences generate transcript variants and /or protein isoforms that vary in structure and function. For example, imagine a drug that suppresses
expression of a specific form of VEGFA, while sparing the other isoforms that control
expression of this gene in healthy tissues. By targeting the specific problematic gene isoform, a
more effective treatment with fewer side effects can be administered. It is not hard to imagine
how specific targeting of individual gene isoforms could lead to more accurate and sensitive
diagnoses as well.

Summary of Research Completed

DEVELOPMENT OF ALGORITHMS FOR RNA-Seq DATA:

NPEBseq: Nonparametric Empirical Bayesian-based Procedure for Differential Expression
Analysis of RNA-seq Data:

NPEBseq Method

NPEBSeq is a nonparametric empirical Bayesian-based approach to model the RNA-seq data.
The expression level of genes with low read counts is estimated by borrowing information from
the gene expression in the whole sample. The non-parametric form of the prior distribution
avoids any unrealistic assumption. The parametric assumption for the prior distribution is
usually not fulfilled for the RNA-seq read count data. The fact that there are many genes
expressed at low levels in one sample is illustrated in Figure 1, which is generated based on one
sample from Marioni’s RNA-seq dataset. This plot clearly shows that a large proportion of
genes in a sample are expressed at low levels. These genes could have a high impact on the
performance of statistical methods to identify differentially expressed genes. The fact that there
are large numbers of genes/transcripts with low read counts and a small number of genes with a
significantly high number of reads make any parametric assumption for the prior distribution
unrealistic.

Performance of NPEBSeq on Simulated Datasets: To evaluate the proposed method for
identifying differentially expressed genes, we first conducted a simulation study.

Simulation 1 - Simulation with different priors between conditions (dataset1):
To generate data similar to those produced by real RNA-seq experiments, we first applied the
empirical Bayes method on publicly available RNA-seq datasets, which were generated to
compare liver and kidney transcriptomes. The prior distributions of kidney and liver samples
were first estimated, and then the data was normalized based on the expected values. The
corresponding dispersion parameter \( \theta \) for each condition was also estimated.

Dataset1 consists of 20 independent simulations, with seven samples each for two conditions.
The library size of each sample is uniformly sampled from 300,000 to 900,000. Each sample
was generated by a mixture of negative binomial model with both the prior distributions and
dispersion parameters estimated from Marioni’s data. Each sample consists of 10,000 genes for
computational efficiency.

We performed a comparative analysis of our method with four popular methods, DESeq, edgeR,
baySeq and NOISeq, which are available as part of Bioconductor packages.
at [http://www.bioconductor.org](http://www.bioconductor.org). The edgeR implements two ways to estimate the dispersion parameter in its model, common dispersion and tag-wise dispersion. Both are studied here. BaySeq provides two choices of model (Poisson and negative binomial). We adopted the negative binomial model for dataset1. Both DESeq and edgeR provide p-values for ranking the genes. BaySeq provides log posterior likelihood ratio for ranking the differential expression of genes. In the case of NPEBseq, we rank the genes by p-values. The purpose of this simulation is to compare the ability of these methods to rank the genes in order of differential expression. The true ranking order of the genes is based on the fold change of differential expression values between the two conditions.

We used the following criteria to compare the performance of different methods. Given a cutoff point \( \tau \) (e.g. the number of genes declared significantly expressed), the efficiency of a statistical method is measured by \( p_\tau \), the expected percentage of the true first \( \tau \) DE genes being correctly declared as the first \( \tau \) DE genes. The average of estimated \( p_\tau \) is calculated from the 20 replicates. The simulation results for dataset1 are shown in Figure 2. The proposed NPEBseq method outperformed other methods.

**Simulation 2 - simulation with the same priors between conditions (dataset2 and dataset3):**
A simulation scheme is applied here to generate dataset2 and dataset3. The library size of each sample was uniformly sampled from 300,000 to 900,000. The prior distribution of \( \lambda \) was assumed to be common between the two conditions and estimated from the liver RNA-seq data of Marioni.

Dataset2 was generated by Poisson distribution and dataset3 by negative binomial distribution, with the dispersion parameter estimated from the liver data. The simulated data consists of 10,000 genes, and one-tenth of those genes were set to be differentially expressed (between condition A and condition B) with \( \lambda_A = b \lambda_B \). In order to produce both over- and under-expression in our simulated data, 500 randomly selected genes were set to have \( b = 4 \) and the remaining 500 genes were set to have \( b = 1/4 \). Both dataset2 and dataset3 consist of 20 independent simulations. Dataset2 was generated without replicates. Similar to dataset1, seven samples per condition per simulation were generated for dataset3. The full ROC curves for dataset2 and dataset3 are shown in Figures 3 and 4, respectively. Based upon examination of these curves, the proposed NPEBseq method appears to perform better than the other methods. To clearly show that NPEBSeq can robustly estimate fold change of genes with low read counts, the estimated fold change of 10 genes from one sample of dataset2 by NPEBseq along with DESeq and edgeR are shown in Table 1. For the cases with zero read count under one condition, DESeq always gives infinite estimation of fold change.

**Real RNA-seq data analysis**
To further evaluate our method, we tested it on two published RNA-seq datasets.

**Real RNA-seq data 1 - Comparison based on one MAQC dataset:**
We first applied NPEBSeq on the MicroArray quality control (MAQC) dataset and compared with DESeq, baySeq, and edgeR. MAQC datasets contain gene expression data from multiple platforms and are extensively used in evaluating different data processing methods. We downloaded the MAQC2 Illumina RNA-seq data from [http://www.ncbi.nlm.nih.gov/sra](http://www.ncbi.nlm.nih.gov/sra), which
contains seven technical replicates of brain reference RNA samples and seven technical replicates of UHR RNA samples. Tophat was used for tag alignment, and counts for each gene were computed by means of HTSeq Python package (http://www-huber.embl.de/users/anders/HTSeq/), using the annotation of the Ensembl genes and only reads that mapped to exons.

As part of the original MAQC project, approximately 1,000 genes were also chosen to be assayed by Taqman qRT-PCR. Those qRT-PCR data were obtained from GEO database, which contains four technical replicates for each of the two samples. The qRT-PCR data were used as a gold standard to benchmark the gene expression values by RNA-seq. We analyzed the qRT-PCR data using the comparative Ct methods. Finally, 407 genes were defined as DE and 119 genes were defined as non-DE. Given the fact that not all the genes were assayed by qRT-PCR, we followed the same procedure that was applied in Bullard’s Evaluation of Statistical Methods for Normalization and Differential Expression in mRNA-Seq Experiments to define the true positive and false positive rates. Given a “DE” or “non-DE” call from qRT-PCR, define a true positive (TP) as the event that the test of interest calls a gene DE that qRT-PCR called DE. A false positive (FP) event occurs when the test calls a gene DE that qRT-PCR called non-DE. The true positive rate (TPR) is defined as

\[
\frac{\text{#TP and qRT-PCR is DE}}{\text{#qRT-PCR is DE}} / \text{total # genes}
\]

and the false positive rate (FPR) is defined as

\[
\frac{\text{#FP and qRT-PCR is non-DE}}{\text{#qRT-PCR is non-DE}} / \text{total # genes}
\]

Note that these are not the standard definitions of TPR and FPR.

qRT-PCR data were annotated by RefSeq. The BioMart R package was used to convert the RefSeq genes IDs for qRT-PCR to Ensembl genes IDs.

The ROC curves from all the compared methods are shown in Figure 5. Clearly, our proposed method has the best performance in terms of sensitivity and specificity.

Real RNA-seq data 2 - Detecting differential usage of exons from RNA-seq data:
We also analyzed the data by Brook et al (Genome Research, 2011), where the effect of the RNAi knockdown of “pasilla” was studied by RNA-seq in the Drosophila melanogaster cell line. The data was downloaded as part of DEXSeq package. The data consists of four control samples and three knockdown samples. The analysis at gene level by NPEBseq reported 107 differentially expressed genes, with nominal FDR control at 0.1 for the comparison of control and knockdown. To assess the specificity of the NPEBseq method, we performed in-condition comparison by making use of the fact that there are four biological replicates in the control group. We applied NPEBseq for the comparison of two control samples versus the other two. NPEBseq reported zero differentially expressed genes with FDR control at 0.1, which indicates that NPEBseq has a very high specificity. We then analyzed Brook’s data at exon level. NPEBseq found differential exon usage for 2,370 counting bins at FDR 0.01 for between-condition comparison and 225 counting bins for within-condition comparison. We also applied
the newest version of DEXSeq on the exon data, which reported 120 counting bins as DE at FDR 0.1. We checked whether NPEBseq and DEXSeq could achieve comparable results by computing the percentage of DE called exons that are common in the two ranked lists of exons generated by both programs. For example, we found that 74 counting bins (exons) were common among the top 120 DE counting bins called by each approach. Further examination revealed that, among the top 120 DE counting bins identified by NPEBseq, 12 were defined as “untestable” by the DEXSeq method due to low read counts in those counting bins. Since the p-value defined in NPEBseq is different from the regular p-value, we didn’t expect these two approaches to report a similar number of DE exons at the same FDR level.

In summary, NPEBseq can be applied to not only detect differential gene expressions from the RNA-seq dataset with technical and biological replicates for both studied conditions, but also to detect differential usage of exons.

Figure 1: Distribution of number of observed reads per gene for genes with read count less than 1000. The number of genes in a RNA-seq dataset is shown in relation to number of mapped reads per gene. X-axis: number of observed reads per gene; Y-axis: frequency of genes.

Figure 2: Simulation results of comparing the performance of DESeq, edgeR and NPEBseq on dataset1. The x axis denotes $\tau$ and y axis denotes $p_\tau$. 
Figure 3: ROC curves based on simulated dataset2. The programs evaluated are: DESeq, edgeR, NPEBseq and NOISeq. The method baySeq is not shown due to its poor performance on dataset without replicates.

Figure 4: ROC curves based on simulated dataset3. The programs evaluated are: DESeq, edgeR, baySeq, NPEBseq and NOISeq.

Figure 5: ROC curves based on MAQC2 real RNA-seq data: Comparison of the performance of DESeq, edgeR, baySeq, NPEBseq and NOISeq methods. We declared non-DE if its RT-qPCR absolute log-ratio was less than 0.2 and DE if its absolute log-ratio was greater than 2.0.

Figure 6: Percentage of DE exons that are common in the two ranked lists of exons generated by NPEBseq and DEXSeq programs. While x-axis denotes the number of declared DE exons and the y-axis denotes the percentage of common calls between both the programs.
Table 1 - Estimated fold change of 10 genes from one sample of simulated dataset2 using NPEBSeq, DESeq and edgeR

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<th>gene_ID</th>
<th>9995</th>
<th>9996</th>
<th>9511</th>
<th>9032</th>
<th>9045</th>
<th>9030</th>
<th>9082</th>
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<th>1</th>
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<tr>
<td>Poisson mean under condition A</td>
<td>0.7741</td>
<td>1.4868</td>
<td>11.5416</td>
<td>424.1334</td>
<td>5.2419</td>
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<td>Poisson mean under condition B</td>
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<td>45.2038</td>
<td>103.8228</td>
<td>1.2832</td>
<td>1.2832</td>
<td>27.4483</td>
<td>0.758</td>
<td>84.8622</td>
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<tr>
<td>TRUE fold change</td>
<td>4</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>observed #reads under condition A</td>
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<td>0</td>
<td>2</td>
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<td>34</td>
<td>13</td>
<td>89</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>observed #reads under condition B</td>
<td>4</td>
<td>2</td>
<td>37</td>
<td>111</td>
<td>0</td>
<td>1</td>
<td>83</td>
<td>4</td>
<td>72</td>
</tr>
<tr>
<td>estimated fc by NPEBseq</td>
<td>2.5813</td>
<td>1.8077</td>
<td>13.2633</td>
<td>3.3515</td>
<td>18.9702</td>
<td>5.8962</td>
<td>1.1168</td>
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<td>estimated fc by DESeq</td>
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<td>inf</td>
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Research Project 2: Project Title and Purpose

*Regulation of Immune Responses in Multiple Myeloma Bone Marrow Microenvironment* – The purpose of this project is to evaluate the contribution of bone marrow immature myeloid cells in insufficient immune responses observed in multiple myeloma and validate strategy aimed to improve anti-tumor immune responses based on targeting these cells.

Anticipated Duration of Project

1/1/2013 – 6/30/2014

Project Overview

The goal of this project is to understand the function of bone marrow immature myeloid cells in multiple myeloma (MM), a blood cancer characterized by uncontrolled growth of malignant plasma cells preferentially in the bone marrow. Using in vivo immunocompetent mouse model of MM we will investigate whether immature myeloid cells expand and accumulate in this disease. We will also evaluate whether these cells are functionally changed in MM. Specifically, the ability of immature myeloid cells to suppress immune responses and thus, become so-called myeloid derived suppressor cells (MDSC), will be determined. Our study will also provide an insight into the kinetic of antigen-specific immune response in MM and investigate the mechanisms by which MDSC could regulate function of immune cells in the bone marrow. Finally, we will determine whether targeting of MDSC could improve anti-tumor immune responses leading to decreased MM burden. The following Specific Aims will be addressed: (1) investigate the involvement of MDSC in regulation of immune responses in MM BM; and (2) determine whether targeting MDSC would improve the anti-tumor immune response in MM. The knowledge gained from this project will be important as it may justify a novel therapeutic approach for treatment of multiple myeloma based on targeting MDSC.
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Other Participating Researchers

None

Expected Research Outcomes and Benefits

This project will address the following questions: Do immature myeloid cells expand in the bone marrow during multiple myeloma progression? Do they acquire an ability to suppress immune responses? What is the mechanism(s) responsible for immunosuppressive activity of immature myeloid cells in multiple myeloma? Is there an antigen-specific T cell response generated in the bone marrow in multiple myeloma and what is the contribution of immature myeloid cells in this immune response? Would targeting immature myeloid cells improve anti-tumor immune responses and decrease tumor burden in multiple myeloma? The answers to these questions will lead to a better understanding the function of immature myeloid cells and the mechanisms of immune suppression in the bone marrow in multiple myeloma. This information will not only provide us with a greater understanding of the immunobiology of this disease, but also may validate a novel therapeutic approach based on targeting immature myeloid cells in multiple myeloma as well as the factors or signaling pathways that mediate the immunosuppressive effect of these cells. If successful, targeting of immature myeloid cells in multiple myeloma would ultimately result in improved anti-tumor immunity and delayed disease progression.

Summary of Research Completed

To understand the role of myeloid-derived suppressor cells (MDSC) in pathogenesis of multiple myeloma (MM), we utilized syngeneic mouse model of this disease. In this model, MM cell lines ATLN and DP42 derived from double transgenic bcl-xL/c-myc mice were injected i.v. into syngeneic mice. As MM cells were homing to the bone marrow (BM), the tumor growth in this model closely resembled human disease.

Initially, we investigated whether MM tumor growth was accompanied by accumulation of MDSCs. In both ATLN and DP42 models, a significant increase in the proportion and absolute number of MDSC in BM was observed as early as one week after tumor cell inoculation (Fig. 1A). From week two, the presence of MDSC in BM was gradually decreased; and, three weeks after tumor cell inoculation, the proportion of MDSC in BM was significantly reduced as MM cells expanded and substituted all hematopoietic cells in BM. In spleen, the proportion and absolute number of MDSCs was also significantly increased one week after tumor cell inoculation. However, this cell population continued to grow during week two post tumor
injection. Only at the end of week three, when mice became moribund and MM cells expanded, the presence of MDSC in spleens declined (Fig. 1B). The proportion and absolute number of MDSC in lymph nodes dramatically expanded by week three (Fig 1C). We next investigated whether MDSCs isolated from BM, spleens, or lymph nodes (LN) of MM-bearing animals acquired an ability to suppress antigen-specific T cell responses during tumor growth. Gr-1+CD11b+ MDSCs were isolated from BM of MM ATLN-bearing mice one or two weeks after the injection of tumor cells by flow sorting using Aria instrument (BD). As a control, Gr-1+CD11b+ immature myeloid cells (IMC) isolated from BM of tumor-free mice were used. The ability of MDSC and IMC to suppress antigen-specific T cell response was tested in ELISPOT and proliferation assays. MDSC and IMC were added to Pmel-1 transgenic splenocytes (responders) in the presence of specific gp100 (EGSRNQDWL) or control peptides. IFN-γ production evaluated by ELISPOT assay and proliferation by [3H]-thymidine incorporation were determined after 48 hours and 96 hours, respectively. MDSC demonstrated potent suppressive activity; whereas IMC did not inhibit T-cell responses (Fig. 1D). Taken together, our data demonstrated that immature myeloid cells possessing immunosuppressive activity accumulated in MM tumor-bearing animals.

We evaluated tumor-specific immune response in the BM and spleens of MM bearing animals. For that, we used DP42 cell line with a stable overexpression of OVA – DP42-OVA. BM and spleen were collected from DP42-OVA bearing mice one, two or three weeks after tumor inoculation. Presence of OVA-specific CD8+ T cells in DP42-OVA-bearing mice was measured using SIINFEIKL-H2Kb pentamers (Fig 2A). After one week, tumor-bearing mice had a presence of pentamer-positive CD8+ T cells in BM as well as in spleens. However, the proportion of pentamer-positive CD8+ T cells in spleens was more than 10-fold lower than in BM indicating that BM was the primary site of accumulation of antigen-specific CD8+ T cells. We also evaluated the presence of IFN-γ+ (Th1-type) CD4+ T cells in MM-bearing mice and were able to detect these cells in BM and spleen one week after tumor inoculation (Fig 2B).
Figure 1. Accumulation and function of MDSC in MM. (A-C) MM tumors were established by i.v. tail vein injection of ATLN or DP42 cells into syngeneic mice. Mice were euthanized at indicated time points after tumor cell inoculation. As a control (naïve), tumor-free mice were used. Proportion (top panels) and absolute number (bottom panels) of Gr1+CD11b+ cells in (A) BM and (B) spleen were detected by flow cytometry using LSR II instrument. (C) Proportion (left panels) and absolute number (right panels) of Gr1+CD11b+ cells in LN were detected by flow cytometry using LSR II instrument. * - statistically significant difference (p<0.05). (D) Gr1+CD11b+ cells were isolated from BM of ATLN-bearing mice one week after tumor cell inoculation and were cultured with splenocytes from Pmel-1 transgenic mice in the presence of control or specific peptides. Proliferation of T cells was measured by [3H]-Thymidine incorporation. Results are mean values obtained for 3 mice with each condition set up in triplicates. ** - statistically significant difference (p<0.001).
Figure 2. Tumor-specific T cell responses in MM. (A,B) DP42-OVA MM-bearing mice were euthanized at indicated time points. (A) BM and spleens were collected and labeled with anti-CD8 antibody and SIINFEKL-H2-Kb pentamer conjugated with FITC and analyzed by flow cytometry. Proportions of ova-specific CD8⁺ T cells in BM and spleens are shown. (B) BM and spleens were collected from DP42-OVA MM-bearing mice. Proportion of IFN-γ⁺CD4⁺ T cells was determined by flow cytometry. Three mice per group were analyzed.

Research Project 3: Project Title and Purpose

Molecular Basis of BRCA1 and PALB2 Tumor Suppression – Our long-term goal is to detail the molecular basis of tumor suppressor activity of breast and ovarian cancer susceptibility genes (BRCA1 and PALB2) in order to develop a more rational and effective therapy following inactivation of these genes in breast and ovarian cancers. We postulate that BRCA1 and PALB2 are transducers of multiple signaling pathways and their functional inactivation in breast and ovarian epithelial cells lead to a loss of responsiveness to extracellular signals. Our working hypothesis is that the tumor suppressor activity of BRCA1 and PALB2 is due to their function as co-activators of transcription for growth inhibitory signals.

Anticipated Duration of Project

1/1/2013 – 6/30/2014

Project Overview

We postulate that breast and ovarian cancer susceptibility genes (BRCA1 and PALB2) are transducers of multiple signaling pathways and their functional inactivation in breast and ovarian epithelial cells lead to a loss of responsiveness to extracellular signals. We envision that the breast and ovarian cancer susceptibility genes function in transcriptional regulation to mediate mammary epithelial cell morphogenesis and differentiation. Our working hypothesis is that the tumor suppressor activity of BRCA1 and PALB2 is due to their function as co-activators of transcription for growth inhibitory signals.
Specific Aims:
1: Determine the molecular basis by which BRCA1 and PALB2 activate transcription in response to NF-kB and RA.

2: Determine how cancer-causing mutations in BRCA1 alter their function at its target genes.

Our studies pinpoint a key role for BRCA1 and PALB2 in transcriptional responsiveness to nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and retinoic acid (RA), an inhibitory signal, in non-transformed breast epithelial cells. In this project, we will assess the mechanism by which BRCA1 and PALB2 mediate activation of NF-kB and retinoic acid responsive genes and dissect the critical domains in BRCA1 and PALB2 required for their function. Our focus will be to understand the functional domains in BRCA1 and PALB2 that are required for transcriptional activation and to dissect the molecular basis for the requirement of such domains. This is particularly critical for the BRCT domain as it may reveal the molecular basis of BRCA1 tumor suppressor activity. All these experiments will be performed using a panel of breast and ovarian cancer cell lines as to insure the generality of our findings with regards to the molecular mechanism of BRCA1 and PALB2.

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Expected Research Outcomes and Benefits

We applied the method of ChIP-sequencing, which was often used in the past for studies of chromatin regulatory complexes to uncover the genomic sites occupied by breast and ovarian cancer genes. This approach had not been tried with breast cancer and fanconi anemia genes and is the unique aspect of our studies. Identification of the genomic sites at which these proteins reside provides a foundation for detailed analysis of their normal function and how their misregulation leads to cellular transformation. Our efforts in combining genomic approaches with functional studies have uncovered a role for these proteins as co-activators of NF-kB and retinoic acid signaling. The application challenges our current thinking regarding the BRCA1 and PALB2 function, by proposing a role for these proteins in regulating activated levels of transcription in response to extracellular signaling important for differentiation and proliferation.
By identifying the exact targets for these cancer genes, we will provide a functional readout (both gene expression changes and possible epigenetic changes) for the assessment of cancer causing mutations in these genes.

Summary of Research Completed

Aim 1: Detailed analysis of BRCA1 and PALB2 in NF-κB function. Toward analysis of aim 1 and achieving the milestones for the first six months, we analyzed the association of BRCA1 and PALB2 with elongating form of human RNAPII and analyzed the co-activation function of BRCA1 and PALB2 in NF-κB responsiveness following stimulation of breast cancer cells with TNF-alpha.

Association of BRCA1 and PALB2 with elongating RNAPII
The chromatin residence of BRCA1 and PALB2, occupying the 5’-end and extending into the body of the transcriptionally active genes, suggested a functional association between BRCA1/PALB2 and the elongating form of RNAPII. Indeed, recent in vitro studies described the association of BRCA1 with the C-terminal domain (CTD) of RNAPII, and the ubiquitination of elongating polymerase by the BRCA1/BARD1 heterodimer. To explore the association of BRCA1 and PALB2 with RNAPII in vivo, we compared our genome-wide occupancy of BRCA1 and PALB2 to an RNAPII ChIP-seq experiment that we previously performed in MCF10A cells using polyclonal antibodies (N-20), which recognize RPB1 independent of its phosphorylation status. Analysis of 373 highly active genes revealed a similar pattern of occupancy for RNAPII and the breast cancer susceptibility proteins (Fig. 1A).

To directly analyze the elongating form of RNAPII, we performed ChIP-seq using antibodies against the Ser2-phosphorylated form of RNAPII (P-Ser2), which corresponds to the elongating form of RNAPII. Genes targeted by BRCA1 and PALB2 display high levels of Ser2 phosphorylation (Fig. 1B). Importantly, the average profile of elongating form of RNAPII is highly similar to that of PALB2 at highly active genes (Fig. 1C), which is accumulating at the 3’ end of genes. Indeed, unbiased clustering of genes occupied by PALB2 and elongating form of RNAPII revealed a strikingly similar profile at all RefSeq genes (Fig. 1C). To assess whether the association of BRCA1 and PALB2 with active genes requires the elongating form of RNAPII, we inhibited transcriptional elongation using Flavopiridol, an inhibitor of RNAPII elongation. Treatment of MCF10A cells with Flavopiridol resulted in concomitant decrease in transcriptional activity as well as the occupancy of BRCA1, PALB2 and elongating form of RNAPII at the 3’-end of all genes tested (Fig. 1D). Taken together, our results point to an intimate functional association of BRCA1 and PALB2 with the elongating form of RNAPII.

The role of BRCA1 and PALB2 in responsiveness to TNF-alpha stimulation
We next examined the role of BRCA1 and PALB2 in TNF-alpha responsiveness in the breast cancer cell line MCF7. Treatment of MCF7 cells with 10 ng/ml TNF-alpha for 1 hour resulted in increased occupancy of p65/RelA, BRCA1 and PALB2 at the promoter of candidate NF-kB target genes (Fig. 2A). Moreover, in contrast to requirement for p65/RelA in the recruitment of BRCA1 and PALB2, depletion of BRCA1 or PALB2 did not affect the occupancy of p65/RelA (Fig. 2B, C). Next we performed gene expression analysis using microarrays to determine the genome-wide responsiveness of MCF7 cells following treatment with TNF-alpha. Overall, 33
genes displayed a significant activation following treatment of MCF7 cells with TNF-alpha (log2(foldChange)>0.4, p<0.01). We validated the response to TNF-alpha using real-time PCR for eight genes in three independent experiments. Additionally, we examined CXCL1, CXCL3, NFKBIA, and SOD2 (which, while responsive to TNF-alpha using real-time PCR, were not reliably detected on the microarray). While depletion of p65/RelA resulted in the loss of TNF-alpha responsiveness in 31 of 33 genes represented on the array and all four additional genes examined (35 of 37 genes examined), BRCA1 depletion led to a diminished responsiveness to TNF-alpha for a subset of genes (18 out of 37; Fig. 2D). A smaller group of genes (10 out of 37) displayed a significant decrease in their response following PALB2 depletion (Fig. 2E). Additionally, a large number of TNF-alpha responsive genes (24 out of 37) showed a significant decrease in their basal activity following depletion of p65/RelA, BRCA1 or PALB2 (Fig. 2D, 2E, p<0.05). It is important to note that infection of MCF10A or MCF7 cells with viral vectors used in our depletion studies may activate a component of the NF-kB pathway, which may contribute to the basal NF-kB responsiveness in MCF10A and MCF7 cells. Taken together, depletion of p65/RelA had a greater effect on TNF-alpha responsiveness than that of BRCA1 or PALB2 depletion, reflecting the fact that there may be other NF-kB co-activators and that BRCA1/PALB2 may only confer a component of the TNF-alpha response.
Figure 1. The breast cancer genes mirror RNA Polymerase II occupancy and are functionally linked with its elongating form.

(A) The average profile of RNAPII, BRCA1 and PALB2 read density of class I genes (n=373) in MCF10A cells. The average read densities of RNAPII peak at the transcription start site and remains elevated across entire gene body similar to the BRCA proteins. (B) Genome-wide analysis of elongating RNAPII was performed in MCF10A cells using antibodies against phosphorylated Ser2 of the CTD (P-Ser2). Snapshots of the aligned-reads for P-Ser2, PALB2, BRCA1 and RNAPII show a compelling overlap between the four ChIP-seq datasets at two loci representative of the target genes of the BRCA1/PALB2 complex. Although spanning the entire gene body, P-Ser2 peaks beyond the 3' end of the gene (multiple isoforms in the case of SOD2), similar to PALB2. (C) Unbiased clustering of PALB2 and P-Ser2 reveals an intimate association between the most recently discovered gene implicated in familial breast cancer and the elongating form of RNA Polymerase II on the left panel (see Fig. S5C for the whole cluster). The right panel depicts the average read profiles of the most actively transcribed genes (comprising of approximately 300 genes). (D) Inhibition of elongation abrogates BRCA1 and PALB2 recruitment at target genes. MCF10A cells were treated with Flavopiridol for 2h and subjected to ChIP analysis at the 3’-end of a group of highly active genes. The stacked bars indicate the residual amount of BRCA1, PALB2, RNAPII and P-Ser2 after flavopiridol treatment, relative to untreated cells (100%). Average of three IPs are shown. A dramatic decrease in elongating RNAPII is mirrored by loss of over 70-80% of the BRCA1-PALB2 complex at chromatin. RT-PCR analysis of the mRNA levels after Flavopiridol treatment is also shown.
Figure 2. BRCA1 and PALB2 affect TNFα-mediated response at NF-kB target genes. (A) ChIP analysis of TNFα-stimulated MCF7 cells shows a substantial recruitment of p65/RelA, BRCA1 and PALB2 at a set of target genes. Cells were analyzed after 1h of treatment. (B) Depletion of BRCA1, PALB2 and p65 in MCF7 cells with lentiviral transduced shRNAs. Due to PALB2 antibodies poorly performing in a total extract, we immunoprecipitated PALB2 before testing its depletion by immunoblot. (C) Impact of BRCA1, PALB2 and p65/RelA depletion on p65/RelA association upon TNF-alpha stimulation in MCF7 cells. ChIP analysis was performed after 1h stimulation with TNF-alpha and reveals that neither BRCA1 nor PALB2 depletion affects p65/RelA binding to the TSS of its target genes. (D) Transcriptional effects of TNF-alpha at selected genes in MCF7 cells. Depletion of BRCA1 but not PALB2 significantly impairs activation in all genes tested (p<0.05) with the exception of NFKBIA (p<0.02). Most genes display attenuation of basal transcription following BRCA1, PALB2 and p65 depletion. (E) Transcriptional effects of TNF-alpha at selected genes in MCF7 cells. Depletion of BRCA1 and PALB2 impairs gene activation mediated by NF-alpha at IL8, PHLDA1, BIRC3 (as resulted from the microarray analysis) and SOD2 (uncovered as a target gene in MCF10A cells). SOD2, IL8 and BIRC3 also show a pronounced attenuation of basal transcription. All four genes displayed a significant attenuation of their responsiveness following depletion of BRCA1 and PALB2 in three independent experiments (p<0.05).
**Research Project 4: Project Title and Purpose**

*Analysis of Markers of Progression and Therapy Resistance in Melanoma* – Melanoma is an aggressive disease for which there is a universally poor prognosis. We have discovered that a dynamic transition of melanoma cells to a mesenchymal phenotype results in a highly invasive and therapy-resistant subpopulation of cells, marked by expression of the protein Wnt5A. This may hold significant implications for novel therapies currently being used to treat melanoma patients, specifically, BRAF inhibitors that are meeting with great success in the clinic, but to which, unfortunately, patients quickly develop resistance. We will test whether inhibiting the Wnt5A signaling pathway can overcome this observed resistance, and elucidate the mechanisms by which Wnt5A contributes to therapy resistance.

**Anticipated Duration of Project**

1/1/2013 – 6/30/2014

**Project Overview**

Melanomas are characterized by a high degree of heterogeneity, invasiveness, and resistance to therapy. Mechanistically, we have shown that the Wnt5a pathway is a critical regulator of mesenchyma in melanoma. We have shown that Wnt5a binds to the receptor tyrosine kinase ROR2, antagonizes canonical Wnt signaling, ultimately conferring a mesenchymal phenotype to melanoma cells. We have recently discovered that this may hold significant implications for novel therapies currently being used to treat melanoma patients. The long-term goal of this research is to define the role of the Wnt5A pathway in therapy resistance.

The BRAF gene is mutated in 50% of melanoma patients, and patients treated with BRAF inhibitors (e.g., PLX4720) demonstrate robust clinical responses, at least for a time. Recently, a requirement for β-catenin and the canonical Wnt pathway for the robust response to BRAF inhibitors has been discovered. These data suggest that the Wnt5A pathway, which inhibits canonical Wnt signaling, might render tumor cells resistant to BRAF inhibitors. To address this we have the following specific aims:

**Specific Aim 1.** Investigate the role of Wnt5A signaling in resistance to BRAF inhibitors. Using genetic and pharmacologic approaches we will manipulate Wnt5a and determine the response to BRAF inhibitors in vitro. We have shown that inhibiting ROR2 in vitro and in vivo will dramatically increase the effects of BRAF inhibitors. We will ask in this aim, whether resistance can continue to occur in the absence of Wnt5A/ROR2, and if so what mechanisms arise to mediate that resistance.

**Specific Aim 2.** Investigate whether Wnt5A induces MAPK signaling via CAMKII. Our preliminary data indicate CAMKII signaling may play a role in Wnt5A mediated resistance to Vemurafenib. We hypothesize that Wnt5A also contributes to increased or at least sustained MAPK signaling, in the presence of BRAF inhibitors, via CAMKII activation. We will ask if MAPK signaling is sustained in resistant cells, and if so if CAMKII is an essential intermediate. We will examine the potential of CAMKII inhibitors as adjuvant therapy to BRAF inhibition.
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Other Participating Researchers
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Expected Research Outcomes and Benefits
Current standard of care for melanoma patients includes the use of inhibitors to the mutant form of BRAF, in patients carrying the BRAF V600E mutation. These drugs have met with initial success, but tumors recur, on average, within 7 months of treatment. The need for improving the efficacy and duration of BRAF inhibitors is critical. We have previously shown that Wnt5A is a key mediator of a switch to a mesenchymal, highly invasive state in melanoma. We have also shown that the novel orphan tyrosine kinase receptor, ROR2, is required for the transduction of non-canonical Wnt signals in melanoma. It is known that canonical Wnt signaling, via β-catenin, plays a key role in the genesis of melanoma, but its role in melanoma metastasis is unclear, with most data pointing to a loss of β-catenin activity in metastatic melanoma. This is consistent with the finding that in some cell systems, Wnt5A can inhibit β-catenin activity, by increasing its degradation. Recently, it has been shown that β-catenin can sensitize melanoma cells to BRAF inhibitors. If Wnt5A can inhibit β-catenin then it follows that Wnt5A positive melanoma cells would be more resistant to BRAF inhibitor therapy.

By understanding how Wnt5A modulates the efficacy of BRAF inhibitors, we will be able to design therapies to sensitize cells to BRAF inhibitor therapies. We will also be able to identify patients who may respond well, vs. those who may not. Successful completion of this aim could have far-reaching implications for current advances in melanoma therapy.

Summary of Research Completed
Overall Progress
In the past six months, we have initiated Aims 1 and 2 as indicated in the original submission. Much of the focus has been on Aim 2, because of exciting pre-clinical data. For Aim 1, we spent the majority of this time making the reagents required to pursue the aims of the grant. To sum up the accomplishments during this reporting period, we have:
Accumulated the vectors required for the BRAF experiments (Aim 1).

Expanded the clinical data which shows that Wnt5A is involved in both acquired and intrinsic resistance (Aim 2).

Demonstrated that Wnt5A can activate MAPK signaling in sensitive melanoma cells and increase their resistance to PLX4720 (Aim 2).

Silenced ROR2 in multiple cell lines in order to identify increases in sensitivity to PLX4720 (Aim 2).

This work, in addition to the preliminary data included in the original submission of this grant, is currently being assembled for publication. The clinical data constitutes one half of a manuscript from our laboratory that is currently in revision for the journal *Cancer Discovery*. Furthermore, the preliminary data and the new data from the tissue biopsies and MTS assays were used to garner an American Cancer Society Institutional Research Grant awarded to our laboratory.

**Detailed Progress**

1. **Accumulation of vectors and creation of lenti-viruses.**

   Generation of Lenti-viral Vectors. To generate lenti-viruses, we are using the Lenti-X system from Invitrogen. Briefly, the Lenti-X plasmid vectors are co-transfected with the Lenti-X-HTX packaging mix into Lenti-X-293 packaging cells (5 X 10^6 cells, in 10 mLs of medium). Virus is collected from the supernatant, cleared of debris using a 0.45 µM filter, and titrated using the Lenti-X qRT-PCR kit, for accurate and reproducible transductions. We created lenti-virus for the tet-inducible BRAFV600E and for the wild type. Next, multiplicity of infection (MOI) will be determined for each cell line. Double-transduced cells will be selected using 1 µg/mL of puromycin, and 500 µg/mL of hygromycin. After double selection, stable transfectants will be characterized for expression of Wnt5A, ROR2, MAPK and β-catenin using western analysis and real time RT-PCR.

2. **Regulation of β-catenin by Wnt5A.**

   We have demonstrated for the first time in melanoma that Wnt5A regulates β-catenin via the E3 ubiquitin Ligase SIAH2. This has previously been shown in developmental systems. Invasive melanoma cells express higher levels of SIAH2 (Figure 1A). Knockdown of Wnt5A reduces the levels of SIAH2 in these lines (Figure 1B). Wnt5A regulates β-catenin (Figure 1C), and requires SIAH2 to do so (Figure 1D). These data indicate that Wnt5A regulates β-catenin in melanoma cells. Since β-catenin promotes sensitivity to BRAF inhibitors, this supports our data that indicate that Wnt5A may promote therapy resistance via the regulation of β-catenin.

3. **Expansion of clinical data.**

   We have significantly expanded our clinical sample staining for the expression of Wnt5A. We have added 13 more samples to the initial 11 that we described in the preliminary data (Figure 2A). Perhaps even more exciting is the fact that we also have 10 paired samples of patient biopsies prior to Vemurafenib treatment and post-relapse. If Wnt5A truly confers clinical resistance to BRAF inhibitors, then we would predict that Wnt5A positive cells might be selected for in patients who relapse (become resistant) while on therapy. To test this, we acquired ten patient samples that had undergone BRAF inhibitor therapy, and for whom we had pre-
therapy and post-therapy relapsed lesions. We scored the levels of Wnt5A expression in these samples, and found that 7/10 post-relapse samples had increased Wnt5A positivity as compared to the pre-therapy lesions (i.e., positivity in a larger percentage of the tumor). Two examples are shown in Figure 2B. In these seven patients, positivity increased from an average of 6% of the tumor cells being positive for Wnt5A pre-therapy to an average of 45% of the tumor cells being positive post-relapse (p=0.016). In the remaining three patients, one sample increased from 25-30% positivity (which we did not consider significant, and therefore scored this unchanged), one remained the same (5% positivity, pre and post) and one actually decreased from 16% positivity to 10% positivity (Table 1). When considering all ten tumors together, the overall increase in Wnt5A staining went from 9% positivity pre-therapy to 36% positivity post-relapse, and was significant by paired T-test analysis at p=0.029.

4. Activation of MAPK signaling by Wnt5A.
We asked whether treatment of sensitive cells with Wnt5A could increase ERK activation; and, indeed, cells treated with Wnt5A have increased PO4- ERK expression (Figure 3A), suggesting that activation of Wnt5A may provide an alternate route to maintaining MAPK signaling in the face of BRAF inhibition. To determine whether this could be the case, we treated Wnt5A low cells with rWnt5A prior to exposing them to PLX4720. Sensitive cells treated with PLX4720 increased their resistance to the drug (Figure 3B). Conversely, knocking down Wnt5A signaling should increase sensitivity to the drug. As shown in our preliminary data, ROR2 knockdown can inhibit Wnt5A signaling. In this progress report we show that treating multiple cell lines with ROR2 siRNA prior to PLX4720 dramatically increases their sensitivity (Figure 3C-F). These data suggest that the Wnt5A pathway plays a role in resistance to BRAF inhibitors, and may represent both a viable target for adjuvant therapy in patients harboring the BRAF mutation as well as a valuable prognostic indicator of therapy response.
<table>
<thead>
<tr>
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<th>% Wnt5A positivity POST RX</th>
<th>Treatment</th>
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</thead>
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<td>90</td>
<td>Vemurafenib/ trematenib</td>
</tr>
<tr>
<td>Patient 2</td>
<td>0</td>
<td>20</td>
<td>Dabrafenib/ trematinib</td>
</tr>
<tr>
<td>Patient 3</td>
<td>0</td>
<td>10</td>
<td>Vemurafenib</td>
</tr>
<tr>
<td>Patient 4</td>
<td>0</td>
<td>90</td>
<td>Vemurafenib</td>
</tr>
<tr>
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<td>0</td>
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<td>Vemurafenib</td>
</tr>
<tr>
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<td>40</td>
<td>Vemurafenib</td>
</tr>
<tr>
<td>Patient 7</td>
<td>20</td>
<td>40</td>
<td>Dabrafenib</td>
</tr>
<tr>
<td>Patient 8</td>
<td>16</td>
<td>5</td>
<td>PLX4032</td>
</tr>
<tr>
<td>Patient 9</td>
<td>5</td>
<td>5</td>
<td>Vemurafenib</td>
</tr>
<tr>
<td>Patient 10</td>
<td>25</td>
<td>30</td>
<td>Vemurafenib</td>
</tr>
</tbody>
</table>

| AVERAGE | 9.1                      | 35.5                       |            |
| T-Test  | p=0.020260               |                            |            |

**Table 1. Expression of Wnt5A During Acquired Resistance.**

Staining of patient samples for Wnt5A pre- and post- therapy indicates that Wnt5A is upregulated in resistant tumor.
Figure 1

A) Q-RT-PCR of invasive melanoma cell lines for SIAH2 mRNA. B) Q-RT-PCR of Wnt5A and SIAH2 after Wnt5A knockdown. C) Treatment of melanoma cells with rWnt5A results in loss of β-catenin, but D) requires SIAH2.

Figure 2

A) Wnt5A positivity correlates to a diminished clinical response to Vemurafenib. B) Staining of patient samples for Wnt5A pre- and post- therapy indicates that Wnt5A is upregulated in resistant tumors (See also Table 1).
Research Project 5: Project Title and Purpose

Developing Rational Strategies for Therapeutic Targeting of NRAS-Mutant Melanomas – Mutations in NRAS are found in approximately 25% of melanomas. These tumors are extremely aggressive and are among the most difficult tumors to treat. Unfortunately, there are no effective therapies to treat patients with NRAS mutant melanomas. Targeting NRAS itself has thus far not been successful; therefore, alternative strategies are necessary. The goal of this project is to identify key molecules that promote survival of NRAS mutant melanomas. These essential molecules could be potential targets for the treatment of NRAS mutant melanomas. We postulate that blocking critical molecules that are essential for survival of NRAS mutant tumors can kill these neoplasms.
Anticipated Duration of Project

1/1/2013 – 6/30/2014

Project Overview

Melanoma, the most lethal form of skin cancer, is caused by genetic mutations in multiple genes, frequently in genes coding for two proteins called BRAF and NRAS. BRAF and NRAS control critical cellular functions through a series of biochemical reactions known as signaling pathways. Despite recent advances treating melanoma, there are no effective therapies for about ~25% of melanomas carrying NRAS mutations. Hence, effective treatments for NRAS mutant melanomas are urgently needed.

An essential requirement to develop effective treatments for NRAS mutant melanomas is to understand the chain of molecular events activated by NRAS. Unfortunately, very little is known about the molecular pathways that are activated by mutant NRAS, and this gap in knowledge hampers the development of effective therapies. Therefore, it is critical to identify the signaling molecules that are activated by NRAS that are essential for tumor survival. This project aims to identify critical signaling molecules activated by NRAS that are essential for survival of NRAS mutant melanomas. Two specific aims are proposed:

Aim 1. Define the molecular consequences of silencing oncogenic NRAS in melanoma. The goals of this aim are: a) to identify human melanoma cells that are dependent of NRAS signaling for proliferation and survival, and b) to determine the biochemical effects of silencing NRAS in melanomas harboring mutations in this oncogene.

Aim 2. Identify genes or pathways that when targeted can potently kill NRAS mutant melanomas. We will evaluate the effect of treating NRAS mutant melanoma cells with drugs that block RAS effector molecules. For these studies we will use cells that have been isolated from melanoma patients and grown in the laboratory.

We expect that our studies will help us identify new drug targets that will help develop effective treatments for melanoma patients.

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Other Participating Researchers

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**Expected Research Outcomes and Benefits**

Melanoma is the most fatal form of skin cancer. The incidence of melanoma has been progressively increasing with over 70,000 melanoma cases diagnosed and close to 9000 deaths every year in the US. Despite recent advances in treating this cancer, the survival rate is less than 5% and the median survival is 6-12 months.

Although NRAS is the second most frequently mutated oncogene in melanoma, there are no therapeutic options currently available for NRAS-mutant melanomas and very little is known about the effector pathways that mediate survival of these tumors. Furthermore, mutations in NRAS correlate with poor clinical outcome and shorter survival. Consequently, effective therapies for NRAS mutant melanomas, which comprise 15-30% of tumors, are urgently needed. Our proposed studies will fill a significant knowledge gap in the field and will have significant clinical and public health implications. Our studies are important because the identification of critical RAS effectors will provide therapeutic targets to control NRAS mutant tumors.

RAS mutations were identified more than three decades ago and are frequently present in a large number of human cancers. Notably, RAS mutant tumors are among the most difficult cancers to treat. Hence, rational treatment options are sorely needed. We anticipate that the proposed studies will have a major impact on the treatment of melanoma and other RAS-driven cancers. The data generated by our studies will be crucial for understanding RAS signaling in melanoma and improving the treatment of this aggressive disease.

**Summary of Research Completed**

Approximately 25% of all melanomas have mutations in the small GTPase NRAS. The NRAS proteins act as molecular switches by transducing extracellular signals from cell surface receptors and activating intracellular signaling cascades that regulate a diverse array of cellular processes.

The most common mutation in NRAS in melanoma is a substitution of glutamine by lysine or arginine in codon 61. This mutation can lead to the constitutive activation of the MAPK and/or PI3K pathways, therefore inducing aberrant cell proliferation and promoting tumor growth. Additionally, the RAS oncogene can also activate an array of other intracellular signaling pathways including the small GTPases Rho, Rac1, and cdc42, the transcription factor NFkB, all of which can induce growth, survival, and invasion of the tumor cells. Therefore, a thorough understanding of the molecular mechanisms underlying NRAS signaling linked to melanoma pathogenesis and drug resistance is critical to devise strategies for therapeutic targeting of NRAS mutant melanomas.

The goal of this project is to determine the molecular consequences of silencing NRAS, aiming to identify signaling molecules or pathways that are essential for survival of NRAS mutant melanomas. Towards this goal, we have identified a panel of seven NRAS mutant melanoma cell lines (Table I). All cell lines are subject to quality control, including microsatellite fingerprinting to verify the identity of each cell line, mycoplasma testing, and genetic validation of NRAS mutation. Further genetic characterization for additional mutations will be done at a
later time.

During this reporting period we have initiated Aim 1a of this project:

To identify human melanoma cells that are dependent of NRAS signaling for proliferation and survival.

Generation of cell lines expressing NRAS shRNA.

1. NRAS shRNA:

Hairpins shRNA targeting human NRAS in lentiviral vectors were obtained from Openbiosystems (ThermoFisher; Table II). These hairpins were originally generated by The RNAi Consortium (TRC) and are now commercially available and already cloned into the lentiviral vector pLKO.1. The pLKO.1 constructs can be packaged into virus particles for use in transduction experiments. Specifically, the replication-incompetent viral particles can be efficiently produced using lentiviral packaging plasmids co-transfected in 293T packaging cells. Transduction of melanoma cells with lentiviruses was conducted according to manufacturer’s guidelines. Briefly, lentiviral NRAS shRNA plasmids were isolated using Invitrogen Maxiprep Kits following the manufacturer’s instructions. To package the lentiviral vectors, 2ug lentiviral plasmids, along with 2ug packaging plasmids consisting of 1:8 ratio of VSV-G vs dr 8.2 packaging plasmids, and 21 ug Arrest-in agent (Openbiosystems) were respectively diluted into 400 ul serum-free DMEM medium. Diluted DNA was then added to the diluted Arrest-in, mixed quickly and incubated at room temperature for 20 minutes. After that, 800ul DNA/Arrest-in complex were applied to 1X10^6 293T cells in 1.2ml serum-free DMEM in a 60 mm dish, and the 293T cells were incubated at 37 °C for five hours. After aspirating the transfection medium, 3 ml DMEM medium containing 10% fetal bovine serum was added, and the transfected 293T was incubated at 37 °C for 48 hours. The lentivirus- containing supernatant were harvested and filtered (using 0.45um low binding protein filters) and stored at -80°C or immediately used.

To transduce cells, lentiviral supernatant was diluted at MOI of 1-5 with medium, then incubated with melanoma cells with 8ug/ml polybrene for 5 hours. After 48 hours of incubation with regular growth medium, the transduced cells were selected with puromycin for 48 hours. NRAS depletion was assessed by Western blotting. None of the hairpins tested efficiently knocked down NRAS (Figure 1). In order to improve the degree of NRAS depletion, we next combined two different hairpins. For these experiments, we infected melanoma cells using two different hairpins simultaneously or sequentially (Figure 2). Combining two hairpins resulted in >90% depletion of NRAS; therefore, all subsequent experiments are performed using two hairpins against NRAS.

2. Silencing NRAS in melanoma.

To evaluate the biological consequences of silencing NRAS in melanoma, we selected a panel of NRAS mutant cells (Table I). All cell lines have been sequenced to determine the NRAS mutation status. During this initial period, we generated five melanoma cell lines expressing NRAS shRNA. Depletion of NRAS in these cell lines was confirmed by immunoblotting;
NRAS levels were decreased by >75% after lentiviral infection in all five cell lines (Figure 3).

To determine the effect of NRAS silencing on cellular growth, we compared relative cell growth in NRAS mutant melanoma cells expressing an shRNA control or NRAS-shRNA. Same number of cells expressing shRNA control or shNRAS (2500-3000 cells) were seeded onto 96-well plates and grown for six days. Relative cell number was determined by MTT assays every 24 hours by normalizing absorbance of NRAS-depleted cells (shNRAS) to the isogenic cell line expressing non-targeting shRNA. We observed that NRAS depletion halted the growth of 2/4 melanoma cell lines (WM3451 and WM852), whereas the other two cell lines evaluated (WM1366 and WM3629) continued to grow, although at a slower rate (Figure 4).

The effect of NRAS depletion was further assessed on cell proliferation by comparing bromodeoxyuridine BrdU incorporation in NRAS mutant isogenic cell lines expressing non-targeting shRNA (control) or NRAS shRNA using a BrdU Cell Proliferation Assay Kit (Cell Signaling Technologies, MA). Briefly, cells were plated at a density of 5,000 to 10,000 cells per well (depending on cell line growth properties) onto 96-well plates and allowed to adhere overnight incubating at 37°C. The following day, the cells were labeled with BrdU for different times, and the plates were then fixed and processed according to the manufacturer's standard protocol. BrdU incorporation was detected using a mouse anti-BrdU antibody. Consistent with the results obtained by MTT assays on cell growth, we found that NRAS depletion led to a decrease in BrdU incorporation with a larger effect in WM3451 and WM852 (Figure 5). Taken together, our preliminary data suggest that not all melanoma cell lines rely equally on oncogenic NRAS for growth and proliferation.

Table I: NRAS mutant melanoma cell lines

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<th>Cell line</th>
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<tr>
<td>M93-047</td>
<td>Q61L</td>
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</tr>
<tr>
<td>WM852</td>
<td>Q61R</td>
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</tr>
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<td>WM4313</td>
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Table II: Hairpins targeting human NRAS (Open Biosystems)

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<td>TRCN0000033257</td>
<td>4</td>
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Figure 1: A melanoma cell line was infected with lentivirus carrying three different shRNA (sh1, sh3, and sh5) against NRAS as described in the text. Transduced cells were selected with puromycin 48h following infection. Puromycin resistant-transduced cells were collected, lysed and analyzed by immunoblotting to determine the efficiency of NRAS knockdown. HSP90 was used as loading control (left panel). Levels of NRAS and HSP90 were quantified using an Odyssey system. Relative quantification is shown in the table on the right.

<table>
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<th>sh3</th>
<th>sh5</th>
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<tr>
<td>NRAS/Hsp90</td>
<td>4.21</td>
<td>3.49</td>
<td>3.95</td>
<td>4.78</td>
</tr>
<tr>
<td>Relative</td>
<td>1.00</td>
<td>0.83</td>
<td>0.94</td>
<td>1.13</td>
</tr>
<tr>
<td>% KD</td>
<td>17.13</td>
<td>6.33</td>
<td>-13.56</td>
<td></td>
</tr>
<tr>
<td>% NRAS</td>
<td>100</td>
<td>82.86</td>
<td>75.30</td>
<td>93.67</td>
</tr>
</tbody>
</table>

Figure 2: A melanoma cell line was infected with viruses carrying two different shRNAs either simultaneously (a) or sequentially (b). Transduced cells were selected with puromycin 48h following infection. Cells were collected, lysed and analyzed by immunoblotting to determine the efficiency of NRAS knockdown. Actin was used as loading control. Levels of NRAS and actin were quantified using an Odyssey system. Relative quantification is shown in the table below.

<table>
<thead>
<tr>
<th>Me1617</th>
<th>control</th>
<th>sh1+3 (a)</th>
<th>sh1+3 (b)</th>
<th>sh2+1 (a)</th>
<th>sh2+1 (b)</th>
<th>sh3+5 (a)</th>
<th>sh3+5 (b)</th>
<th>sh5+2 (a)</th>
<th>sh5+2 (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRAS</td>
<td>1.33</td>
<td>0.08</td>
<td>0.11</td>
<td>0.24</td>
<td>0.08</td>
<td>0.08</td>
<td>0.01</td>
<td>0.28</td>
<td>0.34</td>
</tr>
<tr>
<td>Actin</td>
<td>121.57</td>
<td>79.21</td>
<td>68.33</td>
<td>79.18</td>
<td>75.39</td>
<td>65.91</td>
<td>66.68</td>
<td>72.39</td>
<td>60.16</td>
</tr>
<tr>
<td>NRAS/Actin</td>
<td>1.09</td>
<td>0.10</td>
<td>0.16</td>
<td>0.30</td>
<td>0.11</td>
<td>0.12</td>
<td>0.01</td>
<td>0.36</td>
<td>0.57</td>
</tr>
<tr>
<td>Relative</td>
<td>1.00</td>
<td>0.09</td>
<td>0.15</td>
<td>0.28</td>
<td>0.10</td>
<td>0.11</td>
<td>0.01</td>
<td>0.33</td>
<td>0.52</td>
</tr>
<tr>
<td>% KD</td>
<td>90.77</td>
<td>85.29</td>
<td>72.29</td>
<td>90.30</td>
<td>66.91</td>
<td>98.63</td>
<td>67.17</td>
<td>48.34</td>
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</tbody>
</table>
**Figure 3:** NRAS mutant melanoma cell lines were infected with lentiviruses carrying two different shRNAs. Transduced cells were selected with puromycin 48h following infection. Cells were collected, lysed and analyzed by immunoblotting to determine the efficiency of NRAS knockdown. Actin was used as loading control. Levels of NRAS and actin were quantified using an Odyssey system. Relative quantification is shown in the table below.

![Immunoblot images](image)

<table>
<thead>
<tr>
<th></th>
<th>WM1366 control</th>
<th>WM1366 sh1+3</th>
<th>WM3629 control</th>
<th>WM3629 sh1+3</th>
<th>WM3451 control</th>
<th>WM3451 sh1+3</th>
<th>WM852 control</th>
<th>WM852 sh1+3</th>
<th>WM3060 control</th>
<th>WM3060 sh1+3</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRAS</td>
<td>5.49 (0.21)</td>
<td>36.45 (8.04)</td>
<td>10.78 (0.56)</td>
<td>17.05 (4.19)</td>
<td>15 (2.9)</td>
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<tr>
<td>Actin</td>
<td>75.1 (54.06)</td>
<td>81.94 (90.16)</td>
<td>76.99 (57.81)</td>
<td>105.8 (107.74)</td>
<td>70.97 (80.87)</td>
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</tr>
<tr>
<td>NRAS/Actin</td>
<td>7.31 (0.39)</td>
<td>44.48 (8.92)</td>
<td>14.00 (0.01)</td>
<td>16.12 (3.89)</td>
<td>21.14 (3.59)</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Relative%</td>
<td>1.00 (0.05)</td>
<td>1.00 (0.20)</td>
<td>1.00 (0.00)</td>
<td>1.00 (0.24)</td>
<td>1.00 (0.17)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>% of KD</td>
<td>94.69 (79.96)</td>
<td></td>
<td>99.91 (75.87)</td>
<td></td>
<td>83.03 (80.30)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4:** The effect of NRAS silencing on NRAS-mutant melanoma cells was assessed on cell growth and viability by MTT assays over six days. Relative viability was calculated relative to the isogenic cell line expressing a control shRNA. Data shown as average +/- SEM (n=7).
**Figure 5**: The effect of NRAS silencing on NRAS-mutant melanoma cells was assessed on cell proliferation by BrdU incorporation.