Bryn Mawr College

Annual Progress Report: 2008 Formula Grant

Reporting Period

July 1, 2009 – June 30, 2010

Formula Grant Overview

The Bryn Mawr College received $12,570 in formula funds for the grant award period January 1, 2009 through December 31, 2010. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Analysis of Epigenetic Modifications at Imprinted Loci in Mouse - The majority of genes in mammals are expressed from copies inherited from both the mother and the father, but a small number of genes are only expressed from one of the two copies. The two copies of these genes must be distinguished to properly control expression; the distinguishing mark is referred to as the imprinting mark. For some genes, the imprinting mark has been shown to consist of modifications to the DNA and/or to the proteins DNA wraps around in the cell; such modifications are called epigenetic because they affect DNA structure but not DNA sequence. We plan to analyze epigenetic modifications at the mouse *Rasgrf1* gene, which is expressed solely from the paternal copy in some tissues, but is expressed from both copies in other tissues. This work will further our understanding of which epigenetic modifications are required to regulate *Rasgrf1* expression.

Anticipated Duration of Project

1/1/2009 – 12/31/2010

Project Overview

Genomic imprinting is an unusual phenomenon whereby only one of the two parental alleles is expressed. To achieve this monoallelic expression, the transcriptional machinery must be able to distinguish between the parental alleles and regulate their expression accordingly. Many imprinted genes are associated with imprinting control regions that exhibit differential DNA methylation on the parental alleles. In many cases, the methylated allele is the non-expressed allele, consistent with the proposed role for DNA methylation in chromatin compaction and gene silencing. At the imprinted *Rasgrf1* gene in mouse, DNA methylation of the imprinting control region is associated with the expressed paternal allele in brain. Methylation of this region on the paternal allele appears to serve as the primary imprinting mark, both because it is inherited from sperm and is retained specifically on the paternal allele throughout development. However, the DNA methylation status cannot be the only factor influencing expression of *Rasgrf1*: *Rasgrf1* is

expressed from both the paternal and maternal alleles in several tissues, despite the paternal allele-specific methylation profile. Therefore, there must be additional epigenetic factors that regulate *Rasgrf1* expression.

In order to identify epigenetic modifications specifically associated with expressed *Rasgrf1* alleles, this project will investigate histone modification profiles associated with the paternal and maternal *Rasgrf1* alleles in both monoallelic and biallelic tissues. Histone modifications promoting *Rasgrf1* expression are expected to be detected on the paternal allele in brain, but on both the paternal and maternal alleles in lung and thymus. Conversely, modifications associated with transcriptionally silent chromatin may be detected on the silent maternal allele in brain and would be absent in lung and thymus DNA. In order to analyze histone modifications, we will perform chromatin immunoprecipitation assays. We will precipitate transcriptionally active chromatin from neonatal mouse brain, lung and thymus using antibodies directed against the acetylated forms of histone H3 and H4, then use an allele-specific assay to determine if the DNA associated with these histones is paternal and/or maternal in origin. Similarly, we will immunoprecipitate inactive chromatin using an antibody directed against histone H3K27me3. These experiments will allow us to determine if there is a direct correlation between the histone modification profiles and the expression state of the *Rasgrf1* alleles.

Principal Investigator

Tamara L. Davis, PhD
Associate Professor
Department of Biology
Bryn Mawr College
101 N. Merion Avenue
Bryn Mawr, PA 19010-2899

Other Participating Researchers

Jane Morris - Undergraduate research assistant at Bryn Mawr College

Expected Research Outcomes and Benefits

The human genome contains approximately 25,000 genes, but in any given human tissue only a small number of these genes are expressed. Proper regulation of genes is critical for tissue-specification, growth and development. The regulation of genes is controlled both by DNA sequence elements and epigenetic factors. Epigenetic factors include modifications to the DNA and its associated proteins, the histones. Such modifications alter the interaction between DNA and the histones it wraps around, altering the structure of genes and influencing the ability of genes to be expressed. Previous research has shown that epigenetic modifications are dynamic, and can change in response to the environment. The changeable nature of epigenetic modifications is likely to explain, in part, the onset of disease during the lifespan of an individual; if gene expression patterns change, deleterious consequences may ensue. Therefore, it is crucial to understand the establishment and maintenance of epigenetic modification patterns, and their relationship to gene expression. This project will examine epigenetic modifications at

the mouse *Rasgrf1* gene in different tissues. *Rasgrf1* is expressed solely from the paternal copy in brain, but is expressed from both the paternal and maternal copies in lung. Therefore, *Rasgrf1* serves as an excellent model for investigating the relationship between epigenetic modifications and gene expression patterns. The expected outcome is a detailed analysis of which modifications are associated with *Rasgrf1* when it is being expressed as compared to when it is silent. These data will contribute to our understanding of the role epigenetic modifications play in the growth and development of mammals.

Summary of Research Completed

The goal for this period of the research project was to utilize ChIP methodology to investigate the distribution of modified histones on the maternal vs. paternal alleles of *Rasgrf1* in a monoallelic tissue (such as brain and liver) as compared to a biallelic tissue (such as lung and kidney). ChIP assays allow us to selectively precipitate DNA associated with a particular modified histone form and assess whether the precipitated DNA is maternal *vs.* paternal in origin, hence allowing us to generate a map of histone modifications on the maternal and paternal alleles of *Rasgrf1*.

Preparation of chromatin for use in ChIP assays was described in the Annual Report submitted in July 2009. The protocol we developed to achieve chemical crosslinking between the DNA and its associated histones has not been changed, so the details will not be repeated in this report. However, since July 2009 we have optimized the sonication protocol we use to fragment chromatin into small pieces. All sonications were performed at 4 watts on ice, with 1-2 minutes on ice between sonication pulses to prevent the lysate from becoming too warm. The number of sonication pulses required to achieve optimal fragmentation varied for different tissues. The optimal size range for fragmented chromatin is 200-1000 bp, which we have successfully achieved for liver chromatin using five to eight 20 second sonciation pulses (Figure 1B). We found that chromatin derived from kidney and brain was more resistant to fragmentation. With brain chromatin, we had difficulty obtaining fragments smaller than 5000 bp, even with extended sonication conditions (data not shown). In contrast, we found that we were able to successfully fragment kidney chromatin using seven to ten 20 second pulses (Figure 1D).

Following sonication, we performed ChIP using two different antibodies. The first antibody was directed against an acetylated form of histone H3 (H3ac). This type of histone modification is considered a permissive modification, as it is generally associated with expressed chromatin regions. The second antibody was directed against a form of histone H3 that is methylated at lysine 9 (H3K9me3). This histone modification is considered a repressive modification, as it is generally associated with non-expressed, or silent, chromatin regions. We hypothesized that biallelic tissues, such as kidney, would have H3ac associated with both the expressed paternal and the expressed maternal alleles. In contrast, we expected that monoallelic tissues, such as liver, would have H3ac only on the expressed paternal allele, and would have H3K9me3 on the silent maternal allele.

We analyzed the distribution of the permissive H3ac and the repressive H3K9me3 on the parental alleles in both a monoallelic tissue (liver) and a biallelic tissue (kidney). This protocol varies slightly from the one reported in July 2009. In independent experiments, liver and kidney

chromatin were diluted in 900 µl ChIP Dilution Buffer + protease inhibitors (EZ-ChIP kit, Upstate). The chromatin was pre-cleared by incubating with 60 µl protein G agarose for 1 hour at 4°C, with rotation. The agarose was pelleted by centrifugation at 4000 rcf for 1 minute at 4°C. 10 µl of supernatant was removed and saved as the "input" fraction at 4°C. The remainder of the supernatant was transferred to a fresh tube and incubated with 5 µl of either anti-acetylated histone H3 antibody (H3ac) or anti-trimethylated histone H3 lysine 9 (H3K9me3) overnight at 4°C with rotation. As a negative control, an identical reaction was set up that lacked the antibody; no chromatin was expected to be precipitated in this control experiment. The following day, DNA-protein-antibody complexes were incubated with 60 µl protein G agarose for 1 hour at 4°C with rotation. The complexes were precipitated via centrifugation at 4000 rcf at 4°C for 1 minute. The supernatant, consisting of the unbound fraction, was removed and stored at 4°C. The agarose-antibody-protein-DNA complexes were washed in the following buffers for 3-5 minutes at 4°C with rotation, and complexes were precipitated via centrifugation as described above after each wash: low salt immune complex wash buffer, high salt immune complex wash buffer, LiCl immune complex wash buffer, two washes in Tris-EDTA. The protein-DNA complexes were eluted by incubating in 100 µl Elution Buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 minutes at room temperature; 10 µl of the input fraction was incubated in 200 µl of Elution Buffer. The eluted complexes were separated from the protein G agarose by centrifugation at 4000 rcf for 1 minute, and the supernatant containing the protein-DNA complexes was transferred to a fresh tube. To maximize yield, the elution step was repeated, and the eluates were combined. Crosslink reversal was achieved by adding NaCl to a final concentration of 0.2 M, and incubating at 65°C overnight. The following day, 1 µl 10 mg/ml RNase was added, and the reaction proceeded for 30 minutes at 37°C. To degrade the protein, 10 mM EDTA, 40 mM Tris-HCl and 1 µl proteinase K were added, and incubation proceeded at 45°C for 1-2 hours. Finally, DNA was extracted using 200 µl of a 1:1 mixture of phenol:chloroform and precipitated using 0.3 M NaOAc, 2 volumes of 100% EtOH and 1 µg glycogen. Resulting pellets were resuspended in 20 µl TE and stored at 4°C.

To determine whether the maternal and/or paternal alleles of *Rasgrf1* were associated with H3ac and H3K9me3, we performed the polymerase chain reaction (PCR) on the eluted samples. PCR primers were designed to amplify *Rasgrf1* sequences flanking a strain-specific DNA polymorphism. The presence of the polymorphism allows us to distinguish between maternally-*vs.* paternally-derived products. If we observe both maternal and paternal products following PCR, then the modified histone is associated with both maternal and paternal DNA. In contrast, if we amplify only paternally-derived DNA, we can conclude that the modified histone is only associated with paternal DNA. For all experiments, we performed PCR on the eluted samples as well as on the input material and the unbound, unprecipitated material. In addition, we also performed PCR on the mock-precipitated samples, which should not contain DNA in the eluted fractions.

We first examined the distribution of the permissive modification, H3ac, in liver and kidney chromatin. We successfully amplified PCR product from samples precipitated with H3ac, demonstrating that acetylated histone H3 is found at *Rasgrf1* (Figure 2). We then digested the PCR products with the restriction endonuclease *Ava*II, which cleaves the maternally-derived products into 425 and 125 bp fragments but does not cleave paternally-derived products. As H3ac is a permissive modification, we were expecting it to be associated with the expressed

maternal and paternal alleles in kidney, but hypothesized that it would only be associated with the expressed maternal allele in liver, and would not be found on the silent paternal allele. Contrary to our expectations, we found that H3ac is associated with the *Ras*-DMR on both the maternal and paternal alleles in liver and kidney chromatin (Figure 2). Analysis of a second region contained within the *Ras*-DMR yielded similar results (data not shown).

We next examined the distribution of the repressive modification, H3K9me3 in liver chromatin. *Rasgrf1* is imprinted in liver, such that the paternal allele of *Rasgrf1* is expressed and the maternal allele is silent; therefore, we hypothesized that the repressive H3K9me3 would be found on the maternal allele but not the paternal allele. Preliminary data indicates that, like H3ac, H3K9me3 is associated with both parental alleles (Figure 3).

The ChIP experiments we have conducted thus far have not supported the hypothesis that the parental alleles of *Rasgrf1* have a differential distribution of modified histones consistent with their expression status. However, the experiments we have conducted to date have only examined the distribution of modified histones at the *Ras*-DMR, a regulatory element that is located approximately 30 kb upstream of the *Rasgrf1* promoter. We are therefore interested in examining the distribution of histones closer to the *Rasgrf1* transcriptional start site. To this end, we have examined the sequence in a region flanking the *Rasgrf1* promoter for polymorphisms that will allow us to distinguish between the maternal and paternal alleles in our F_1 hybrid mice. We have recently obtained preliminary data suggesting the presence of a polymorphism that would introduce a *Dpn*I restriction site in one of the two strains we use to generate the hybrid mice.

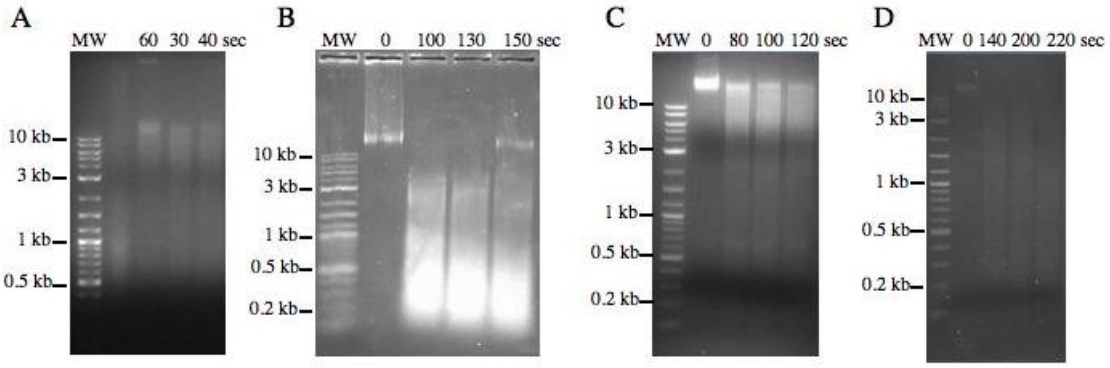

Figure 1. Optimizing sonication conditions for chromatin. (A) Sonication of liver chromatin with a 3.2 mm probe for 30-60 seconds achieved minimal fragmentation, as evidenced by the smear of DNA fragments around 10 kb. (B) Sonication of liver chromatin for 100-150 seconds sheared the bulk of the chromatin into 200-1000 bp fragments. (C) Sonication of kidney chromatin for 80-120 seconds was not optimal. (D) Sonication of kidney chromatin for 140-220 seconds sheared the chromatin into 200-3000 bp fragments. MW = molecular weight marker.

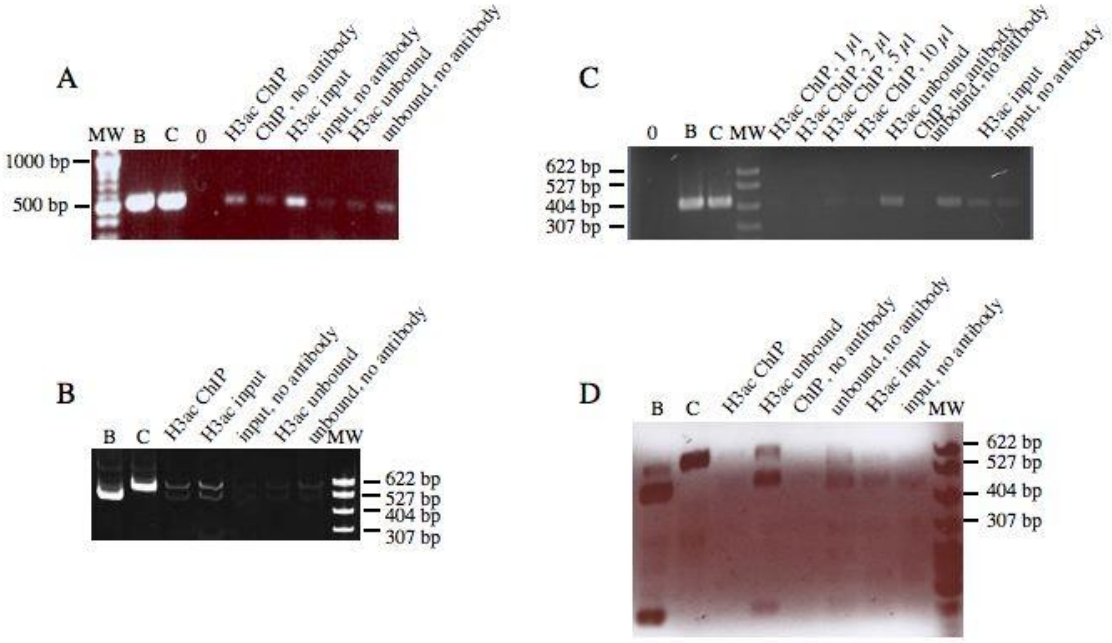

Figure 2. ChIP using anti-H3ac, a permissive modification. (A) PCR amplification of the *Ras*-DMR from input, unbound and precipitated liver chromatin. The expected size of the PCR product is 550 bp. (B) Restriction digestion of PCR products from (A); both B and C products are observed, illustrating that both maternal/B and paternal/C chromatin is associated with H3ac. (C) PCR amplification of the *Ras*-DMR from input, unbound and precipitated kidney chromatin. (D) Restriction digestion of PCR products from (B); both maternal/B and paternal/C chromatin is associated with H3ac.

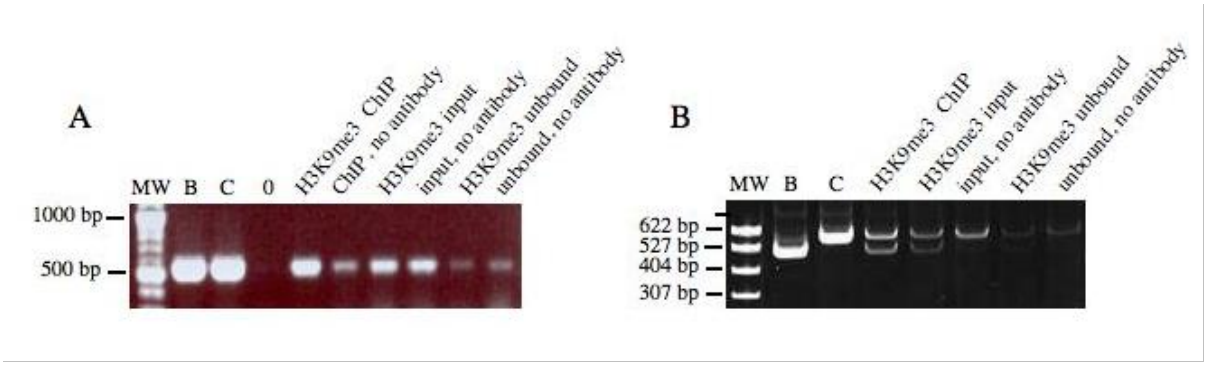

Figure 3. ChIP using anti-H3K9me3, a repressive modification. (A) PCR amplification of the *Ras*-DMR from input, unbound and precipitated liver chromatin. The expected size of the PCR product is 550 bp. The band in the ChIP, no antibody lane is likely a result of contaminants present in the sample; additional washes should eliminate this band. (B) Restriction digestion of PCR products from (A); both maternal/B and paternal/C products are observed, illustrating that both maternal and paternal chromatin is associated with H3K9me3.