Bryn Mawr College

Annual Progress Report: 2008 Formula Grant

Reporting Period

July 1, 2010 – December 31, 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.

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.

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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 of our research is to investigate how epigenetic modifications function to regulate the expression of imprinted genes, which are only expressed from one of the two parental alleles. The work that has been funded by this grant has focused on *Rasgrf1*, a tissue-specific imprinted gene. *Rasgrf1* is imprinted in brain and liver tissue, where it is expressed solely from the paternally inherited allele; in other tissues, such as lung and kidney, *Rasgrf1* is not imprinted, and expression derives from both the paternally inherited and the maternally inherited alleles. Previous research has illustrated that DNA methylation is restricted to the paternal allele in tissues displaying either imprinted or biallelic expression of *Rasgrf1*, suggesting that other epigenetic factors must play a role in regulating the tissue-specific imprinting of this gene. Based on the hypothesis that histone modifications may be involved in controlling tissue-specific imprinting, we have examined the distribution of permissive and repressive histone modifications at the *Rasgrf1* locus.

In order to examine histone modification patterns associated with *Rasgrf1*, we are utilizing chromatin immunoprecipitation (ChIP) assays. 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*. The goal for this period of the research project was to utilize ChIP methodology to complete the on-going analysis of histone modifications associated with the *Rasgrf1*-DMR in kidney chromatin and to investigate the distribution of modified histones on the maternal and paternal chromosomes at the *Rasgrf1* promoter.

One method we used to prepare chromatin for use in ChIP assays, sonication, was described in the Annual Reports submitted in July 2009 and July 2010. Due to on-going technical problems we have had with the consistency associated with chromatin sonication, during this period of the research project we began using an alternative method to prepare the chromatin: micrococcal nuclease. Micrococcal nuclease is an enzyme that cleaves DNA between nucleosomes and can be used to fragment chromatin into nucleosomal units. We utilized a Chromatin Preparation Module (Pierce Thermo Scientific) to fragment liver and kidney chromatin with micrococcal nuclease. Prior to micrococcal nuclease treatment, we homogenized tissues and crosslinked the DNA to histones as described in our previous reports. Following the quenching of unreacted formaldehyde with 0.125 M glycine, cells were pelleted via centrifugation at 5000 rpm for 5 minutes at room temperature, washed two times in 10 ml of 1x phosphate buffered saline (PBS), and washed two times in 1 ml of 1x PBS + protease inhibitors. Cells equivalent to 50 mg of tissue were lysed in 500 µl membrane extraction buffer (Chromatin Preparation Module, Pierce

Thermo Scientific). The resulting nuclei were collected by centrifugation at 9000 rpm for 3 minutes. Nuclei were resuspended in micrococcal nuclease digestion buffer, and incubated in the presence of micrococcal nuclease for 15 minutes at 37°C. The micrococcal nuclease was inactivated via the addition of 20 mM EGTA, and the fragmented chromatin was released by incubating the nuclei in nuclear extraction buffer for 15 minutes on ice. An image illustrating the optimization of micrococcal nuclease digestion can be found in Figure 1. Based on these results, we chose to use 0.5 units of micrococcal nuclease to digest the chromatin isolated from 10 mg of tissue.

Following micrococcal nuclease digestion and chromatin extraction, 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 predicted that in monoallelic tissues, such as liver, H3ac would be associated with the expressed paternal allele, and H3K9me3 would be associated with the silent maternal allele.

To complete the set of investigations described in the July 2010 Annual Report, we analyzed the distribution of the repressive H3K9me3 on the parental alleles of the *Rasgrf1*-DMR in kidney. Details of the methodology utilized can be found in the July 2010 Annual Report; modifications to this protocol follow. Briefly, kidney chromatin that had been fragmented with micrococcal nuclease was diluted and incubated with anti-H3K9me3 overnight at 4°C with rotation. As a negative control, an identical reaction was set up with anti-rabbit IgG, which is not expected to react with mouse histones. The following day, the antibody-protein-DNA complexes were incubated with 20 µl protein A/G agarose for 1 hour at 4°C with rotation. The agarose-antibody-protein-DNA complexes were washed as described in the previous report. Protein-DNA crosslinks were reversed and proteins were degraded via a 90 minute incubation at 65°C in 0.2 M NaCl and 0.2 mg/ml proteinase K. To recover the eluted DNA, samples were diluted in 750 µl DNA binding buffer and centrifuged through a DNA Clean-Up Column (Pierce Thermo Scientific), the column was washed with 750 µl of DNA Column Wash Buffer and eluted in 50 µl DNA Column Elution Solution.

To determine whether the maternal and/or paternal alleles of the *Rasgrf1*-DMR were associated with H3K9me3 in kidney chromatin, we performed the polymerase chain reaction (PCR) on the recovered immunoprecipitated samples, as well as on the input material, the IgG-precipitated samples, and control BL/6 and *castaneus* DNA (Figure 2A). The resulting PCR products were digested with *Ava*II, which cleaves maternally-derived BL/6 products, but leaves paternally-derived *castaneus* products intact. We found that H3K9me3 was associated with the *Rasgrf1*-DMR on both the maternal and paternal alleles, as evidenced by the presence of both cleaved and uncleaved products in the H3K9me3 lane (Figure 2B). This result is consistent with the data presented in the July 2010 Annual Report, and indicates that the distribution of the permissive H3ac and the repressive H3K9me3 is identical on the parental *Rasgrf1*-DMR alleles in liver and kidney, despite the differences in *Rasgrf1* expression in these two tissues.

We next examined the distribution of the two modified histones at the *Rasgrf1* promoter, which we predicted might directly reflect the expression status of this gene. In order to perform an allele-specific analysis of the *Rasgrf1* promoter products that we obtained via ChIP, we needed to identify a strain-specific DNA sequence polymorphism that would allow us to distinguish maternally-inherited DNA from paternally-inherited DNA. Accordingly, all of our experiments were conducted using F_1 hybrid tissues derived from crosses between BL/6 and *castaneus* mice, and sequence polymorphisms between these two strains allowed us to distinguish maternally-inherited DNA from paternally-inherited DNA. To identify polymorphisms, we sequenced approximately 2 kb of genomic DNA from BL/6 and *castaneus* mice and compared the sequences. We identified several DNA sequence polymorphisms, one of which introduced a *Dpn*II restriction endonuclease recognition site in the *castaneus* sequence. Therefore, restriction digestion of PCR products flanking this polymorphism would result in a 370 bp product derived from BL/6 DNA, while the *castaneus* products would be 244 + 126 bp in length.

Liver and kidney chromatin fragmentation and immunoprecipitation with anti-H3ac and anti-H3K9me3 were carried out as described above. PCR was performed on the resulting immunoprecipitated products using primers flanking the *Dpn*II polymorphism identified near the promoter (Figure 2C). The resulting PCR products were digested with the restriction endonuclease *Dpn*II. We found that both the maternal and the paternal alleles of the *Rasgrf1* promoter are associated with H3ac and H3K9me3 in liver and kidney (Figure 2D).

The ChIP experiments we have conducted thus far have not supported the hypothesis that the parental alleles of *Rasgrf1* have differential distribution of modified histones consistent with their expression status. We were concerned that this result might reflect a lack of sufficient chromatin fragmentation, therefore we analyzed an imprinted gene, *H19*, at which the expressed maternal allele is enriched for H3ac and the silent paternal allele is enriched for H3K9me3. While the liver chromatin data are inconclusive, the data obtained from kidney chromatin illustrate an enrichment of the maternal BL/6 allele in the H3ac ChIP sample, and a corresponding enrichment of the paternal *castaneus* allele in the H3K9me3 ChIP sample (Figure 3). We therefore believe that our methodology is working and that there are not striking differences in the distribution of H3ac or H3K9me3 on the maternal and paternal alleles of *Rasgrf1* in liver and kidney chromatin.

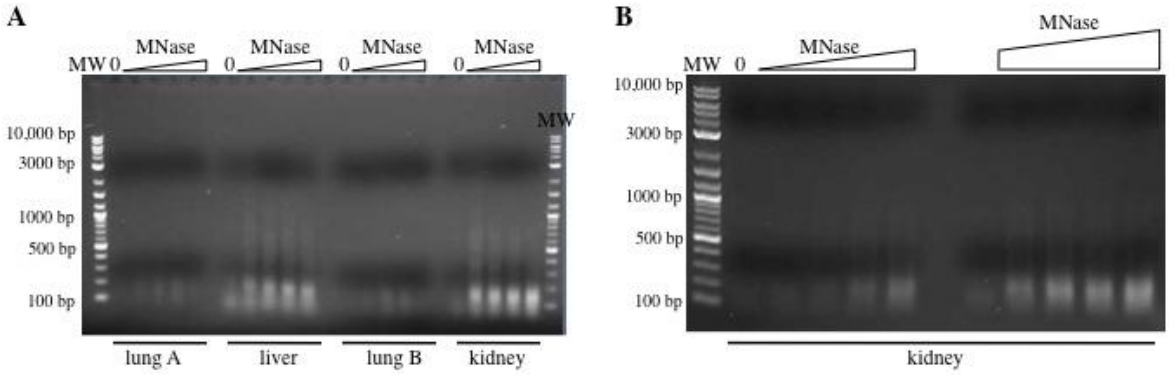

Figure 1. Optimizing chromatin fragmentation using micrococcal nuclease digestion. (A) Lung, liver and kidney chromatin were treated with increasing concentrations of micrococcal nuclease (MNase). Amounts of MNase used: 2.5, 5, 7.5 and 10 units. Mononucleosomes of 160 bp DNA are visible as bright bands just above the 100 bp marker; notice that larger fragments of DNA are visible at lower MNase concentrations. (B) Kidney chromatin was treated with increasing concentrations of MNase: 0.25, 0.5, 1, 2.5, 5, 7.5 and 10 units. Notice that fewer mononucleosomes are visible at lower MNase concentrations.

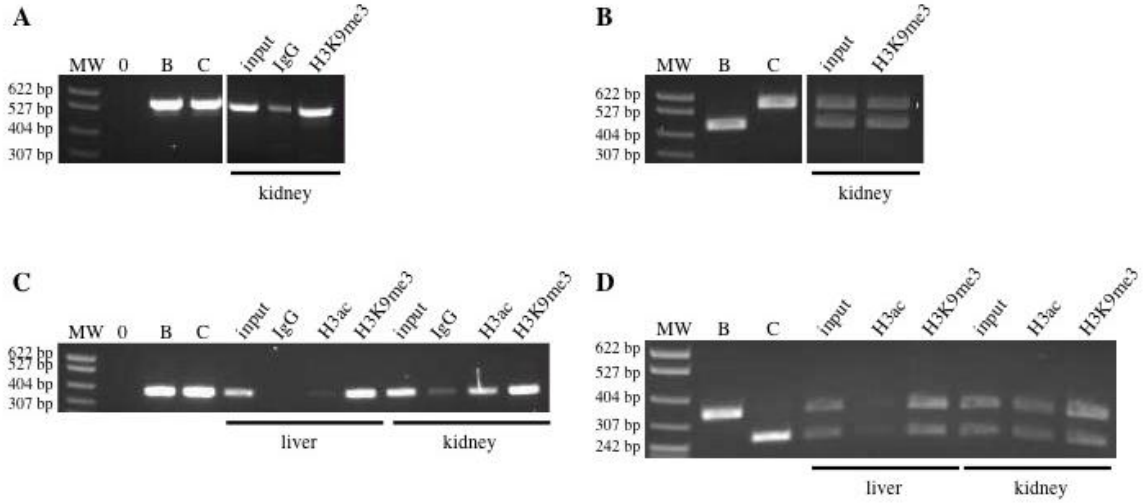

Figure 2. ChIP of liver and kidney chromatin, using anti-H3ac, a permissive histone modification, and anti-H3K9me3, a repressive histone modification. (A) PCR amplification of the *Rasgrf1*-DMR from control BL/6 and *castaneus* DNA, and ChIP input, IgG and anti-H3K9me3 from kidney chromatin. The expected size of the PCR product is 550 bp. (B) Restriction digestion of PCR products from (A); both BL/6 and *castaneus* products are observed in the kidney chromatin samples, illustrating that both maternal and paternal chromatin is associated with the repressive marker H3K9me3 in both a tissue exhibiting biallelic expression of *Rasgrf1* (kidney). (C) PCR amplification of the *Rasgrf1* promoter following ChIP of liver and kidney chromatin with anti-H3ac or anti-H3K9me3; the expected size of the PCR product is 370 bp. (D) Restriction digestion of PCR products from (C); both BL/6 and *castaneus* products are observed in all liver and kidney chromatin samples, illustrating that both maternal BL/6 and paternal *castaneus* chromatin is associated with the permissive marker H3ac and the repressive marker H3K9me3 in both a tissue with imprinted expression of *Rasgrf1* (liver) and a tissue with biallelic expression of *Rasgrf1* (kidney).

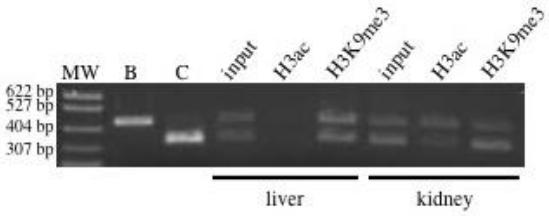

Figure 3. ChIP analysis of liver and kidney chromatin at the *H19* locus. PCR products derived from the *H19* differentially methylated region were digested with *Dpn*II, which cleaves products derived from the paternal *castaneus* chromosome into 300 + 98 bp fragments; the 398 bp maternal BL/6 product remains intact. Note an increase in maternally-derived BL/6 product in the H3ac kidney sample, and an increase in the paternally-derived *castaneus* product in the H3K9me3 kidney sample.