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DNA Methylation in Genomically Imprinted Genes of Mice

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Abstract

Many questions in genetics are raised toward the causes and effects of imprinted genes. A major exception to Mendel's laws, imprinted genes possibly have effects in the speciation of mammals and in biomedical phenomena like mental disorders and cancers. A common explanation for the silencing for one parental allele but not the other is differential methylation in regions of imprinted genes called imprinting control regions (ICR). A loss of this methylation in the cytosine residues of cytosine-guanine dinucleotides (CpG) can perhaps explain the hybrid inviability and disorders mammals experience. This investigation examined the ICRs of four known imprinted genes (Grb10, Mest, Peg10, and Zim2) in *Mus musculus, Mus domesticus* and their hybrid offspring for possible losses in methylation. We discovered for the paternally expressed gene Peg10, the ICR is indeed differentially methylated, but this methylation is conserved in all F_1 offspring.

Introduction

Genomic imprinting is an epigenetic phenomenon that results in the unequal gene expression of alleles based on the parent of origin. It is considered a major exception to the Mendelian laws of inheritance (Vrana et al., 2000). This mechanism evolved around the time monotremes, marsupials, and eutherians last shared a common ancestor (180-210 million years ago) (Hore, Rapkins, & Graves, 2007). The main theory aimed at explaining the benefit of genomically imprinted genes is the kinship hypothesis. The kinship hypothesis proposes that genomic imprinting evolved as a result of a conflict between maternal and paternal genes over growth of

offspring. Paternal genes allowed for increased growth of the offspring, which would require more of the mother's resources than smaller offspring. This is detrimental to the mother, but beneficial to the father since stronger offspring will result in a higher probability of the father's genes being passed on to the next generation (Tilghman, 1999). Therefore, maternally silenced genes usually result in increased growth, and paternally silenced genes result in smaller offspring (Vrana, Guan, Ingram, & Tilghman, 1998). Considering the similarities between genomes of all mammals, it is also known that imprinting genes are highly conserved (Pai, Bell, Marioni, Pritchard, & Gilad, 2011). Figure 1 attempts to diagram genomic imprinting.

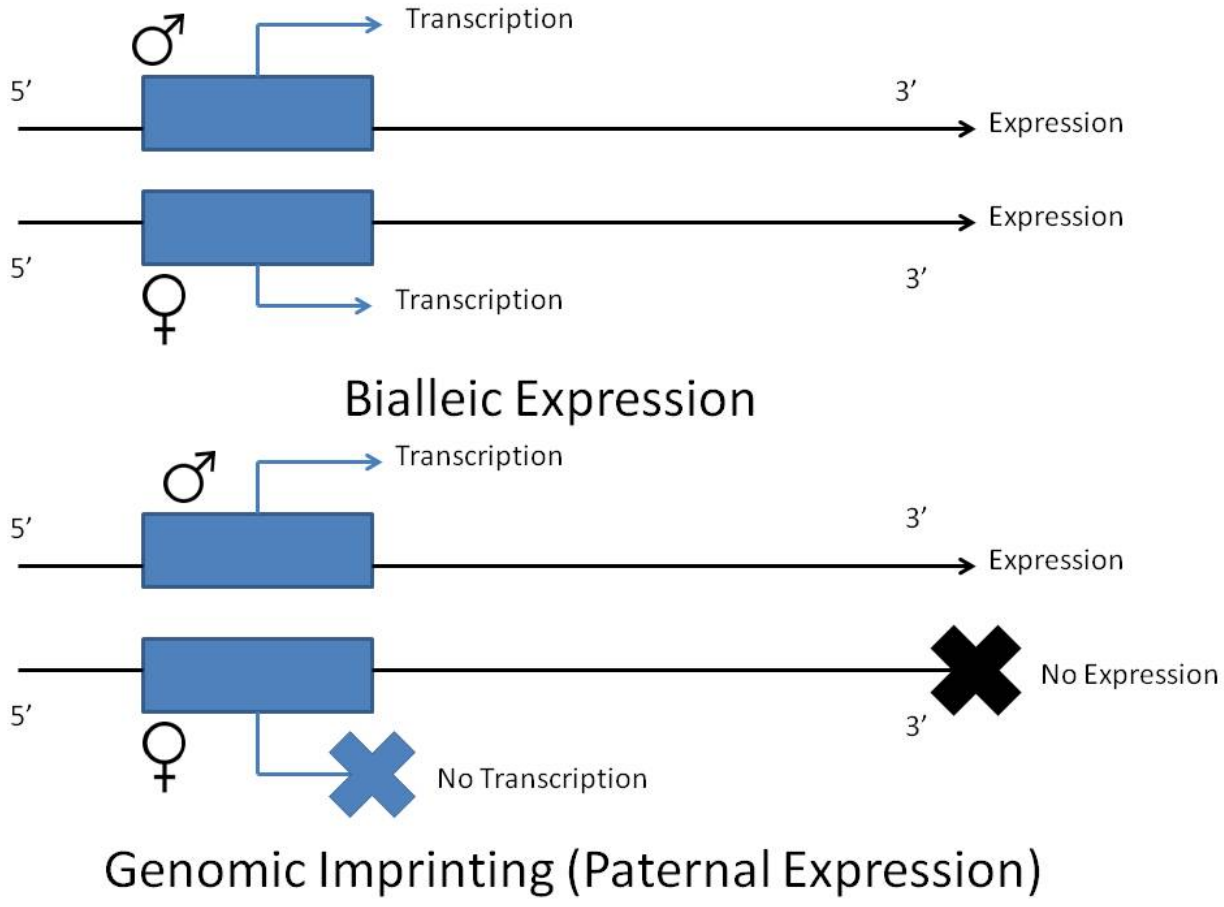

Figure 1. Diagram depicting genomic impriniting.

A loss of imprinting (LOI) may result in an undergrowth or overgrowth of offspring, and is observed in hybrids of both *Peromyscus* (Vrana et al., 2000) and *Mus* (Shi et al., 2004). Loss of imprinting has also been found to be responsible for placental dysplasia, and hybrid inviability (Shi et al., 2004). The effects LOI have on hybrids results in a postzygotic barrier to speciation in *Peromyscus* and *Mus*, and perhaps other mammals. A commonly hypothesized explanation for the loss of imprinting is that a loss of methylation in a silenced allele will result in expression of that allele, resulting in either overgrowth or undergrowth of offspring. This theory is supported by the fact that in known imprinted genes, complete methylation is commonly observed in their imprinting control regions (ICRs) (Tilghman, 1999). Methylation at a promoter site prevents the allele from being transcribed by not allowing the transcription factors to bind (Tilghman, 1999).

DNA methylation is when a methyl group is added to a cytosine residue of DNA, and commonly occurs on C-G dinucleotides (CpG). Figure 2 shows a methylated cytosine. Methylation plays a role in X-chromosome inactivation and susceptibility to cancers, as well as genomic imprinting (Pai et al., 2011). Bisulfite conversion is a popular method used to find methylated cytosines. The conversion works by converting unmethylated cytosines to uracils by removing an amino group and replacing it with a carbonyl group. Methylated cytosines are protected from this reaction, so once the reaction is sequenced, methylated cytosines can be found. Figure 3 displays a methylated cytosine residue before and after bisulfite conversion. This allows for known imprinted genes to be converted and sequenced in order to find differences in imprinted genes between hybrids *Mus musculus, Mus domesticus*, and their hybrids.

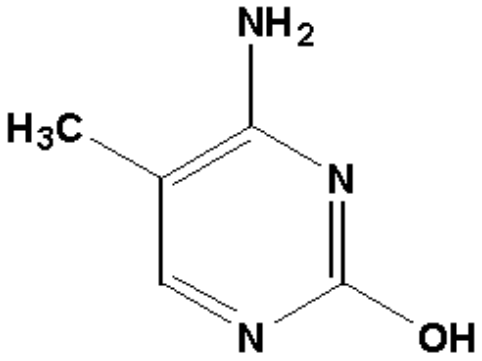

Figure 2. A methylated cytosine. Image obtained from Pearson Higher Education.

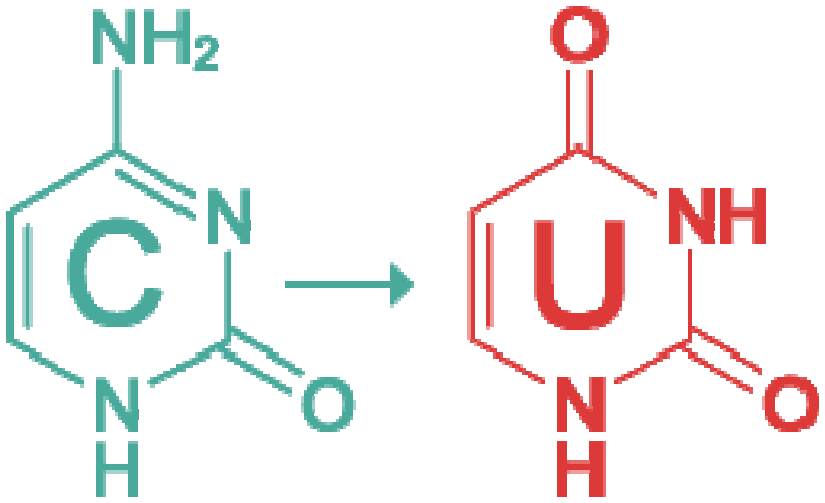

Figure 3. Conversion of an unmethylated cytosine to a uracil by bisulfite conversion. Image obtained from Zymo Research.

The five genes that will be examined in this study are Grb10, Mest, Peg10, and Zim2. Grb10 is a gene that is in a family with Grb7 and Grb14, commonly referred to as adapter proteins. Standing for growth factor-receptor bound protein 10, Grb10 is maternally expressed in mice. Disruptions in the maternal allele of this gene result in overgrowth of offspring (Charalambous et al., 2003). In humans, Grb10 is located on the short arm of chromosome 7, as opposed to chromosome 11 in mice. It is believed that in humans, Grb10 may be linked to Silver-Russell Syndrome (SRS),

since maternal disomies of human chromosome 7 are responsible for the growth retardation experienced in SRS. In humans, however, Grb10 is biallelically expressed throughout the body, except for the fetal brain, where it is paternally expressed. In mice, Grb10 is maternally expressed throughout the body, except for where it is biallelically expressed in the brain. In mice, maternal duplication of chromosome 11, where Grb10 is located, results in prenatal growth retardation, but paternal duplication of chromosome 11 is response for a promotion in growth (Hikichi, Kohda, Kaneko-Ishino, & Ishino, 2003). The Grb10 protein itself binds to active tyrosine kinase receptors that are responsible for growth in mice.

Mest, also known as Peg1 (paternally expressed gene 1), is an abbreviation for mesoderm specific transcript. It is a gene located on mouse chromosome 6, and is paternally expressed throughout the body (Reule, Krause, Hemberger, & Fundele, 1998). In humans, Mest is located on chromosome 7 (Lefebvre, Viville, Barton, Ishino, & Surani, 1997). While the function of the Peg1/Mest protein is not known for sure, it is believed that Mest does play a role in cancers, especially breast cancer (Pedersen et al., 1999), as well as infertility in men (Poplinski, Tüttelmann, Kanber, Horsthemke, & Gromoll, 2010).

Peg10, which stands for paternally expressed gene 10, is located on mouse chromosome 6, and human chromosome 7. It is a retrotransposon derived gene that highly conserved among mammals, and is necessary for development of the placenta, individuals without the Peg10 are inviable, despite the fact Peg10 is retrotransposon derived (Ono et al., 2006). This paternally expressed gene is highly expressed in hepatocellular carcinomas, and may be involved in other cancers as well (Okabe et al., 2003).

Zim2 is a mostly paternally expressed gene located on mouse chromosome 7. In humans it is closely linked with another paternally expressed gene, Peg3. Both Zim2 and Peg3 share a set of seven exons that compose the 5' ends of both genes. In humans, Zim2 and Peg3 are located on chromosome 19. In mice, Zim2 is also expressed bialleically in the testes and maternally in the brain, but paternally expressed elsewhere in the body (Kim, Bergmann, Lucas, Stone, & Stubbs, 2004). Zim2 codes for a zinc finger protein, which is a protein that has incorporated zinc ions into its structure.

For this study, we will be utilizing *Mus musculus* and *Mus domesticus,* two species of the house mouse that last shared a common ancestor 350,000 years ago. Figure 4 shows the appearance of *Mus.* Both these are the same species as lab mice. A natural and well-studied hybrid zone of these two species forms a narrow band that stretches across Europe, which is displayed in figure 5. Hybrid males of *M. musculus* and *M. domesticus* are often sterile, and both sexes harbor more parasites than either of their parent's species (Payseur & Nachman, 2005). We will be using wild-derived strains of these mice, and all of the genetic tools developed for lab mice can be used with these strains.

Figure 4. *Mus musculus*, the common lab mouse. Image obtained from the Jackson Laboratory

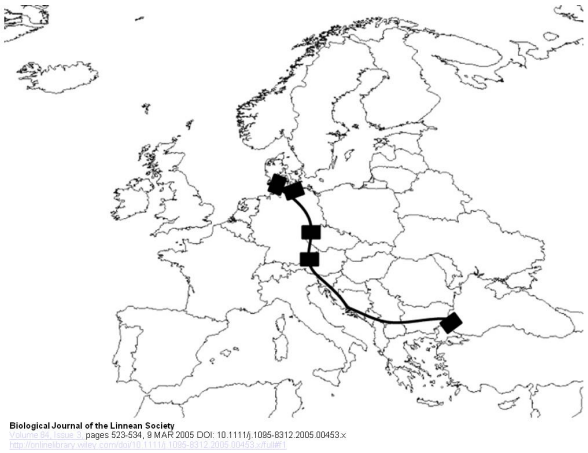

Figure 5. The natural *Mus musculus/ Mus domesticus* hybrid zone. The line represents the hybrid zone, while the boxes are areas that have been studied. Image obtained from Payseur & Nachman, 2005.

This investigation aims to improve our understanding of genomically imprinted genes and DNA methylation. We hypothesize a loss of methylation will be observed in the ICRs for the four loci in each of the four hybrid mice we are examining.

Methods

DNA Samples

Genomic DNA samples for each *Mus* species (*Mus musculus, Mus domesticus, Mus musculus* x *Mus domesticus, and Mus domesticus* x *Mus musculus*) were obtained from the lab of Dr. Bret Payseur, University of Wisconsin-Madison, and leftover from a previous investigation conducted by Tori LeFleur.

Bisulfite Conversion

Using a Thermo Scientific NanoDrop 2000c Spectrophotometer, the DNA concentration of each sample was determined by measuring the absorbance of each sample at 260 nm. A mass of 400 ng of DNA then underwent bisulfite conversion using a Zymo Research EZ DNA Methylation Kit. Protocol used matched the recommended protocol provided with kit.

Primer Construction

Primers sequences used for the amplification of bisulfite converted DNA were obtained from the literature of Hikichi et al. for Grb10, Hiura et al. for Mest, and Lucifero et al. for Zim2. Primers sequences used for the amplification of bisulfite converted Peg10 were determined using the Bisulfite Primer Seeker Tool by Zymo Research. Primers used in the amplification of genomic DNA were found with help from the Primer3 online primer design tool. Primer sequences used are detailed in Table 1.

Table 1. Sequences of primers used in DNA amplification

Gene	Direction	Sequence
Genomic Grb10	Forward	5'-CAACCGCTGTCTACCACTTG-3'
	Reverse	5'-CGTATGTTGGCGCGTGTT-3'
Genomic Peg10	Forward	5'-CTTGGCCAGTTCAGCATCG-3'
	Reverse	5'-GATTCCTCTGCGATGCCAC-3'
Genomic Zim2	Forward	5'-CCGAGGCCTGGACCTATAGA-3'
	Reverse	5'-GGGGAATGGGGTCTTGGATT-3'
Bisulfite Converted Grb10	Forward	5'-GAGAAGATATGTTGAAGTTATGGTG-3'
	Reverse	5'-TAAATACAATTACTACTTATTACATAATATC-3'
Bisulfite Converted Mest	Forward	5'-TTTTAGATTTTGAGGGTTTTAGGTTG-3'
	Reverse	5'-TCATTAAAAACACAAACCTCCTTTAC-3'
Bisulfite Converted Peg10	Forward	5'-TTGGYGTTTTTTTTTTTAGGATTTTTTTATATAAGG-3'
	Reverse	5'-AAAAAATCCTAACCATACTCACCACAC-3'
Bisulfite Converted Zim2	Forward	5'-TTTTGTAGAGGATTTTGATAAGGAGG-3'
	Reverse	5'-AAATACCACTTTAAATCCCTATCACC-3'

DNA Amplification

DNA amplification was conducted according to the protocols outlined in Figure 2 using a Bio-Rad MyCycler Thermal Cycler. GoTaq polymerase was used to amplify genomic DNA and ZymoTaq Pre-mix was used to amplify bisulfite converted DNA.

Table 2. PCR protocol

Gene	Initial Denature	Denature	Anneal	Extension	Final Extension	Cycles
Genomic Grb10	4 min, 95 C	30 sec, 95 C	30 sec, 51 C	30 sec, 72 C	7 min, 72 C	30
Genomic Peg10	4 min, 95 C	30 sec, 95 C	30 sec, 48 C	30 sec, 72 C	7 min, 72 C	30
Genomic Zim2	4 min, 95 C	30 sec, 95 C	30 sec, 48 C	30 sec, 72 C	7 min, 72 C	30
Bisulfite Converted Grb10	10 min, 95 C	30 sec, 95 C	30 sec, 45 C	30 sec, 72 C	5 min, 72 C	35
Bisulfite Converted Mest	10 min, 95 C	30 sec, 95 C	30 sec, 48 C	30 sec, 72 C	5 min, 72 C	35
Bisulfite Converted Peg10	10 min, 95 C	30 sec, 95 C	30 sec, 48 C	30 sec, 72 C	5 min, 72 C	35
Bisulfite Converted Zim2	10 min, 95 C	30 sec, 95 C	30 sec, 48 C	30 sec, 72 C	5 min, 72 C	35

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to determine if amplification of DNA was successful as well as to purify the PCR product. A 1% agarose gel was used in each electrophoresis, with

ethidium bromide as the visualization agent. A volume of 5 µL loading dye was added to each PCR product before the entire 30 µL solution underwent electrophoresis. In addition to the PCR product, 5 µL exact Gene 100bp PCR DNA Ladder was included to determine product size. Each gel was run at 100 V for approximately 60 minutes before it was visualized using a Bio-Rad Gel Doc XR+ Molecular Imager. Figure 6 shows an image of a successful DNA amplification once gel electrophoresis is complete.

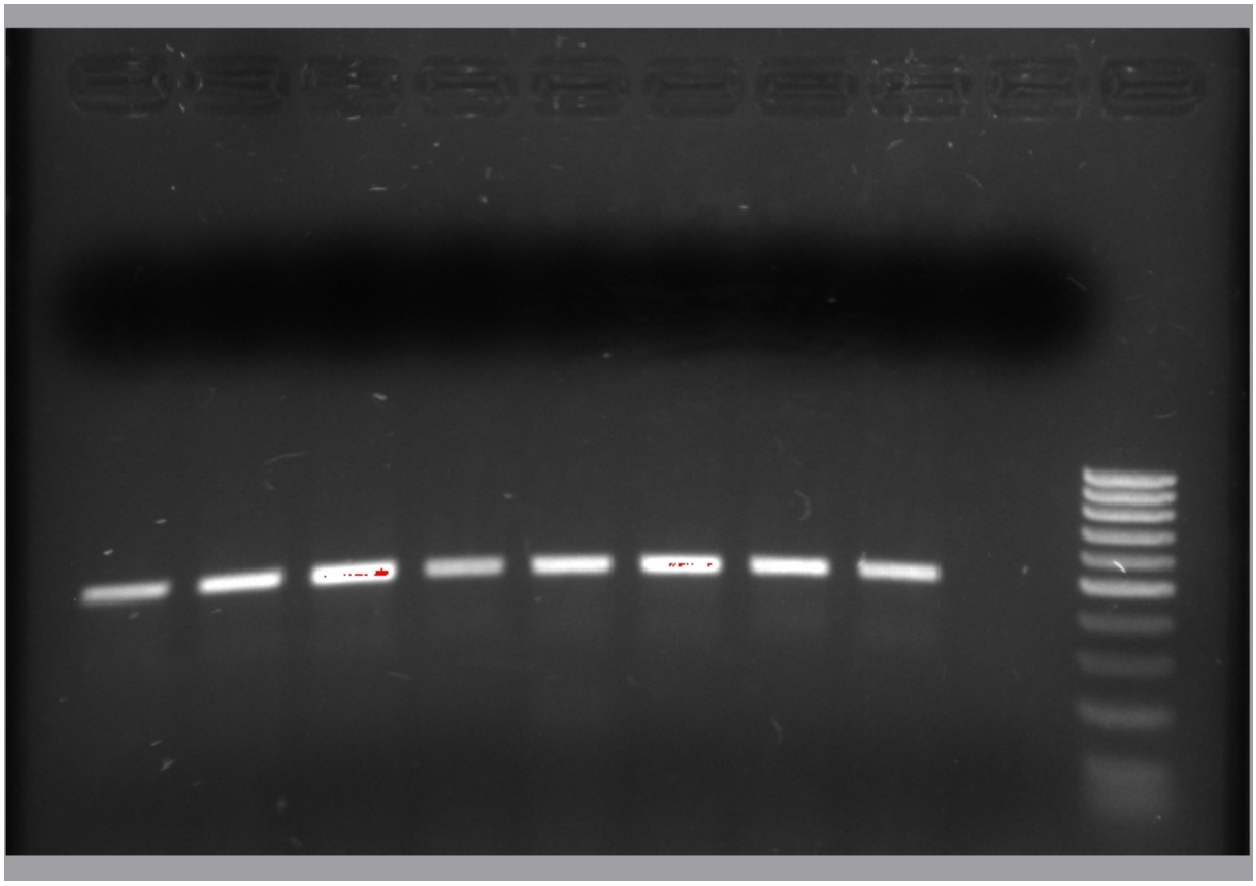

Figure 6. 1% agarose gel in 1x TBE. This gel displays the successful amplification of genomic Zim2, with mice samples in lanes 1-8, negative control in lane 9, and 100 bp DNA ladder in lane

Gel Extraction

PCR product was extracted from the agarose gels using a QIAquick Gel Extraction Kit produced by Qiagen. Protocol matched the protocol recommended by Qiagen. All genomic DNA samples, as well as bisulfite converted Grb10, were suspended in Qiagen buffer TE, and bisulfite converted Mest, Peg10, and Zim2 were suspended in distilled water.

Sequencing

All samples were sent to Genewiz, Inc. of South Plainfield, NJ for sequencing. Two 96-well plates were prepared according to guidelines provided by Genewiz, with 10 µL of approximately 1 ng DNA/µL gel extracted PCR product and 5 µL of primer at a 5 µM concentration. Absorbance at 260 nm was measured using a Thermo Scientific NanoDrop 2000c spectrophotometer in order to determine the concentration of DNA in the gel extracted PCR product. Chromatophores obtained for each DNA sample from Genewiz were then analyzed using Geneious bioinformatics software.

Results

Grb10

Using the Basic Local Alignment Search Tool (BLAST) to verify the identity of genomic DNA sequences obtained, it was confirmed that genomic Grb10 was amplified and sequenced. However, after performing an in silico bisulfite conversion of the known ICR of Grb10 and aligning the results with the consensus sequence obtained for bisulfite converted Grb10, it was discovered that a bisulfite converted ICR for Grb10 was not amplified and sequenced, but instead an unknown loci was amplified. With no way to determine the true identity of the

sequenced loci due to the bisulfite conversion, analysis of both Grb10 and the unknown loci was unable to be conducted.

Mest

After using BLAST to confirm the identity of the genomic Mest sequenced, it was found that genomic Mest was not amplified, but a different ICR for a gene named Mcts2 was instead sequenced due to an error when designing primers. Since the genome for the common lab mouse has been sequenced, the known sequence for the ICR was used as a reference rather than the experimental ICR that we attempted to amplify and sequence. In a bid to expand the project, bisulfite converted Mcts2 was then sequenced, but the quality of the sequences returned were too poor to use in our analysis of methylation.

Upon comparing the consensus sequence obtained for bisulfite converted Mest to the in silico bisulfite conversion of the known ICR for Mest, it was once again determined a different bisulfite converted loci was amplified and sequenced rather than Mest, preventing us from making any conclusions concerning the methylation patterns in Mest.

Peg10

Entering the consensus sequence obtained for genomic Peg10 into BLAST produced Peg10 as a top hit, confirming that the genomic loci amplified was indeed Peg10. Performing an in silico bisulfite conversion of this sequence also confirmed that the bisulfite converted Peg10 sequence obtained in the experiment was actually the bisulfite converted ICR for Peg10. Aligning the consensus bisulfite converted ICR sequence for Peg10 with its consensus genomic ICR sequence (Figure 7) further proved that the desired regions of DNA were sequenced, with differences

between the two limited to where cytosines in the genomic sequence were converted to thymines in the bisulfite converted sequence.

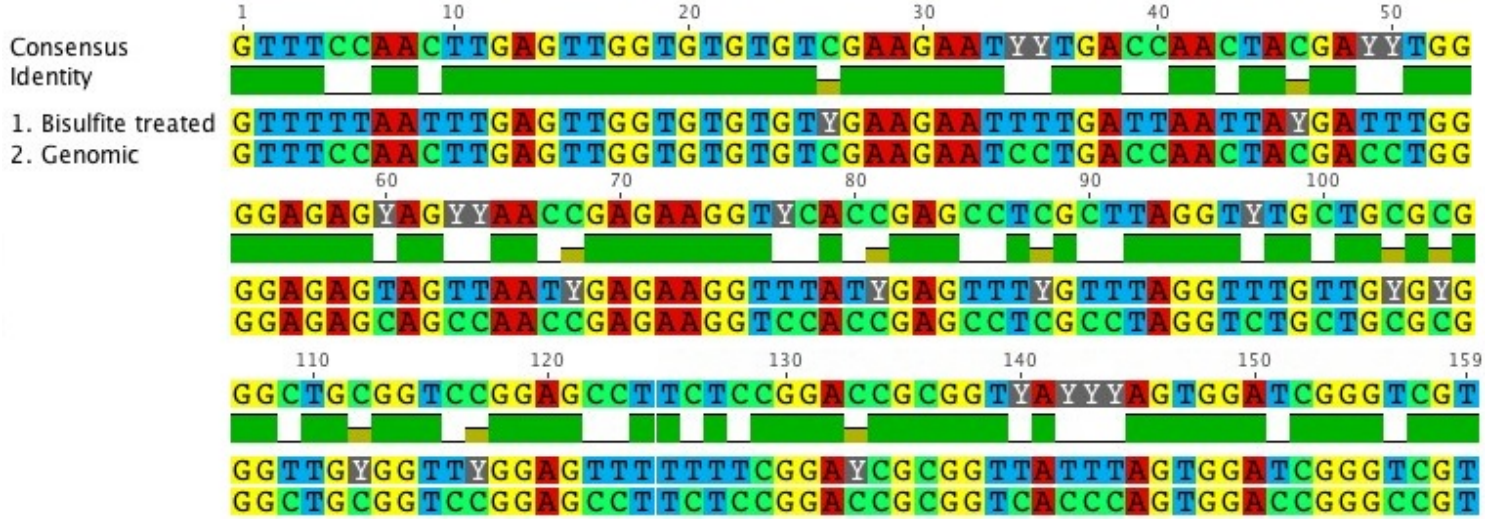

Figure 7. Comparison between bisulfite converted Peg10 and genomic Peg10.

Full sequencing results are shown in figures 9A and 9B, along with their comparisons to the other mouse individuals. A total of 14 CpG sites were analyzed in the sequence that is roughly 160 base pairs long for each individual. Figure 8 shows a simplified diagram depicting the CpG sites examined.

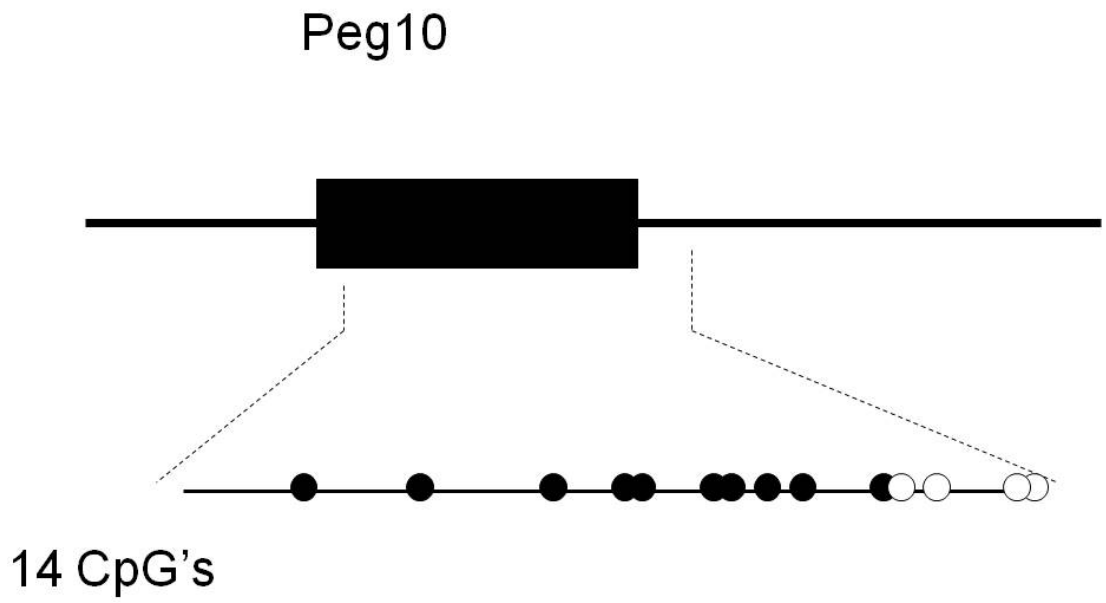

Figure 8. Depiction of CpG sites relative to placement on Peg10. The box represents the ICR of Peg10, while the circles on the zoomed in strand represent CpG sites. Shaded in circles represent a CpG site where a C-T heterzygote is present, while open circles represent where regular CpG residues are present on both the genomic and bisulfite converted Peg10.

Peg10 *M. m. musculus* female:

TTTTAATTTGAGTTGGTGTGTGTYGAAGAATTTTGATTAATTAYGATTTGGGGAGAGTAGTTAATY
GAGAAGGTTTATYGAGTTTYGTTTAGGTTTGTTGYGYGGGTTGYGGTTYGGAGTTTTTTTCGGAYC
GCGGTTATTTAGTGGATCGGGTCGTCGCGGGATTT

Peg10 *M. m. domesticus* male:

TTTTAATTTGAGTTGGTGTGTGTYGAAGAATTTTGATTAATTAYGATTTGGGGAGAGTAGTTAATY
GAGAAGGTTTATYGAGTTTYGTTTAGGTTTGTTGYGYGGGTTGYGGTTYGGAGTTTTTTTCGGAYC
GCGGTTATTTAGTGGATCGGGTCGTCGCGGGATTTT

Peg10 F_1 Female (*M. m. musculus* x *M. m. domesticus*):

TTTTTAATTTGAGTTGGTGTGTGTYGAAGAATTTTGATTAATTAYGATTTGGGGAGAGTAGTTAAT
YGAGAAGGTTTATYGAGTTTYGTTTAGGTTTGTTGYGYGGGTTGYGGTTYGGAGTTTTTTTCGGAY
CGCGGTTATTTAGTGGATCGGGTCGTCGCGGGATTTTTT

Peg10 F_1 Male (*M. m .musculus* x *M. m. domesticus*)

GTTTTTAATTTGAGTTGGTGTGTGTYGAAGAATTTTGATTAATTAYGATTTGGGGAGAGTAGTTAA
TYGAGAAGGTTTATYGAGTTTYGTTTAGGTTTGTTGYGYGGGTTGYGGTTYGGAGTTTTTTTCGGA
YCGCGGTTATTTAGTGGATCGGGTCGTCGCGGGATTTTTT

Figure 9A. Sequence comparison between a *M. musculus* female, a *M. domesticus* male, and their hybrid offspring. Yellow highlighting indicates heterozygous CpG sites, where Y represents a C-T heterozygote. Gray highlighting indicates non-heterozygous CpG sites.

Peg10 *M. m. domesticus* Female:

TTTTAATTTGAGTTGGTGTGTGTYGAAGAATTTTGATTAATTAYGATTTGGGGAGAGTAGTTAATY
GAGAAGGTTTATYGAGTTTYGTTTAGGTTTGTTGYGYGGGTTGYGGTTYGGAGTTTTTTTCGGAYC
GCGGTTATTTAGTGGATCGGGTCGTCGCGGGATTTT

Peg10 *M. m. musculus* Male:

TTTTAATTTGAGTTGGTGTGTGTYGAAGAATTTTGATTAATTAYGATTTGGGGAGAGTAGTTAATY
GAGAAGGTTTATYGAGTTTYGTTTAGGTTTGTTGYGYGGGTTGYGGTTYGGAGTTTTTTTCGGAYC
GCGGTTATTTAGTGGATCGGGTCGTCGCGGGATTT

Peg10 F_1 Female (*M. m. domesticus* x *M .m. musculus*):

TTTTAATTTGAGTTGGTGTGTGTYGAAGAATTTTGATTAATTAYGATTTGGGGAGAGTAGTTAATY
GAGAAGGTTTATYGAGTTTYGTTTAGGTTTGTTGYGYGGGTTGYGGTTYGGAGTTTTTTTCGGAYC
GCGGTTATTTAGTGGATCGGGTCGTCGCGGGATTTTTT

Peg10 F_1 Male (*M. m. domesticus* x *M. m. musculus*):

GTTTTTAATTTGAGTTGGTGTGTGTYGAAGAATTTTGATTAATTAYGATTTGGGGAGAGTAGTTAA
TYGAGAAGGTTTATYGAGTTTYGTTTAGGTTTGTTGYGYGGGTTGYGGTTYGGAGTTTTTTTCGGA
YCGCGGTTATTTAGTGGATCGGGTCGTCGCGGGATTTTTT

Figure 9B. Sequence comparison between a *M. domesticus* female, a *M.musculus* male, and their hybrid offspring. Yellow highlighting indicates heterozygous CpG sites, where Y represents a C-T heterozygote. Gray highlighting indicates non-heterozygous CpG sites.

At various base pairs in the sequences, C-T heterozygotes were expected to be seen, since one allele is expected to be methylated within the ICR, while the other allele is expected to be unmethylated. This results in the fluorescence of both T and C residues for selected CpG islands in the sequence of Peg10. The two peaks of fluorescence were used to identify 10 differentially methylated base pairs in the sequence of Peg10. An example of a heterozygote in the chromatogram is included in Figure 10.

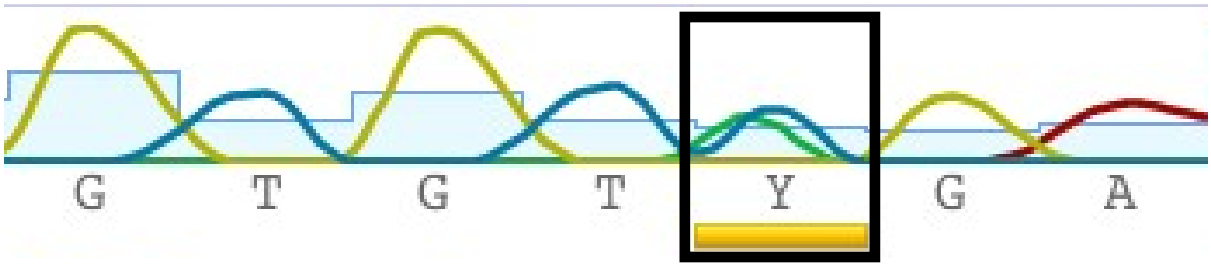

Figure 10. Example of a C-T heterozygote in the chromatogram following bisulfite conversion. The heterozygote, which is outlined in black in the figure and labeled with "Y," has both a blue peak for the tyrosine residue present on one allele, and a green peak for the cytosine residue present on the other allele.

Zim2

Similar to the sequences obtained for both Grb10 and Mest, the sequence obtained for genomic Zim2 was determined to be accurate after using BLAST. Also, it was found that the bisulfite converted Zim2 sequence obtained during the experiment was not the desired sequence after alignment with a sequence obtained using an in silico bisulfite conversion of the known sequence for the ICR of Zim2. This discovery prevents us from making any conclusions regarding Zim2.

Discussion

The amplification of undesired loci instead of Grb10, Mest, and Zim2 leaves Peg10 as the only loci available for analysis, since it is currently not possible to discover which loci were amplified in place of the desired ones, due to the bisulfite conversion. For future use, this suggests that designing primers from scratch may be more advantageous than borrowing them from literature, since all of the failed loci were amplified using primers chosen from literature. Despite the issues this investigation, Grb10, Mest, and Zim2 are all loci worth investigating in the future due to the volume of literature and research done concerning these loci.

Peg10

Looking at the sequences returned for Peg10 in all eight individuals (Figures 9A and 9B), there are fourteen total CpG sites in each individual. Of the fourteen, the first ten contain C-T heterozygotes between the two alleles for each specimen, and the last four CpG sites do not contain heterozygous C-T residues. This suggests that the first ten CpG sites are within the DMR for Peg10, while the final four CpG sites happen to be outside the DMR. This also suggests that within the DMR for Peg10, one allele is completely methylated, while the other allele is completely unmethylated, which was expected.

Focusing on the regions of the sequences that are known to be within the DMR of Peg10, it is evident that the sequences for these regions are identical for all eight individuals examined, including at the heterozygous CpG sites. The conservation in the bisulfite converted sequences between hybrid offspring and their parents shows that there is no loss of methylation in the DMR for Peg10 in the hybrid offspring of *M. musculus* and *M. domesticus*.

Peg10's role in the development of the placenta plays a large role in the viability of offspring. Peg10 could possibly be partly responsible for the fetal overgrowth and placental dysplasia experienced in inviable offspring. Since there is no loss of methylation in the hybrid offspring of mice, however, the resultant under- or overexpression of Peg10 that would accompany a loss in methylation cannot be responsible for the placental defects that cause the offspring to perish. Deletions in the Peg10 can still be responsible for placental defects, but a loss in methylation in Peg10 cannot alone be responsible. The same applies for the overexpression of Peg10 that been found in some cancers.

The development concerning the conservation of methylation of Peg10 in hybrid offspring also poses questions concerning speciation. Even though there is a small natural hybrid zone in Europe (Figure 5) where hybrid mice survive, it appears as though the hybrid mice are inviable elsewhere where both *M. musculus* and *M. domesticus* coexist. Since methylation and imprinting have been shown to be conserved for Peg10, this suggests there may be other barriers to speciation preventing the viability of mouse hybrids in these areas.

The findings concerning Peg10, however, should not be applied to imprinted genes as a whole. It is known that other genomically imprinted genes do experience a loss in methylation and imprinting, making LOI still a possible explanation for phenomena like hybrid inviability and speciation. Future investigations concerning imprinting genes in mice and their hybrids should include more individuals and strains in order to increase genetic diversity of the samples. Other *Mus* species besides *M. musculus* and *M. domesticus* would also provide a more complete understanding of methylation in mice. Furthermore, future studies should also attempt to explain

the effects LOI have specifically on organ systems and individuals, to better understand the functions of genomically imprinted genes. A better understanding of these genes will help to improve future research and methods of biomedical phenomena affected imprinted genes like Silver-Russell Syndrome, Angelman Syndrome, Prader-Willi Syndrome, cancers, and in vitro fertilization.

In conclusion, our findings that methylation is conserved in Peg10 of the hybrid offspring of *M. musculus* and *M. domesticus* do not support our hypothesis that methylation in genomically imprinted genes of mouse hybrids is lost.

References

Charalambous, M., Smith, F. M., Bennett, W. R., Crew, T. E., Mackenzie, F., & Ward, A. (2003). Disruption of the imprinted Grb10 gene leads to disproportionate overgrowth by an Igf2-independent mechanism. *Proceedings of the National Academy of Sciences*, *100*(14), 8292–8297. doi:10.1073/pnas.1532175100

Hikichi, T., Kohda, T., Kaneko-Ishino, T., & Ishino, F. (2003). Imprinting regulation of the murine Meg1/Grb10 and human GRB10 genes; roles of brain-specific promoters and mouse-specific CTCF-binding sites. *Nucleic Acids Research*, *31*(5), 1398–1406. doi:10.1093/nar/gkg232

Hore, T. A., Rapkins, R. W., & Graves, J. A. M. (2007). Construction and evolution of imprinted loci in mammals. *Trends in Genetics*, *23*(9), 440–448. doi:10.1016/j.tig.2007.07.003

Kim, J., Bergmann, A., Lucas, S., Stone, R., & Stubbs, L. (2004). Lineage-specific imprinting and evolution of the zinc-finger gene ZIM2. *Genomics*, *84*(1), 47–58. doi:10.1016/j.ygeno.2004.02.007

Lefebvre, L., Viville, S., Barton, S. C., Ishino, F., & Surani, M. A. (1997). Genomic Structure and Parent-of-Origin-Specific Methylation of Peg1. *Human Molecular Genetics*, *6*(11), 1907–1915. doi:10.1093/hmg/6.11.1907

Okabe, H., Satoh, S., Furukawa, Y., Kato, T., Hasegawa, S., Nakajima, Y., … Nakamura, Y. (2003). Involvement of PEG10 in Human Hepatocellular Carcinogenesis through Interaction with SIAH1. *Cancer Research*, *63*(12), 3043–3048.

Ono, R., Nakamura, K., Inoue, K., Naruse, M., Usami, T., Wakisaka-Saito, N., … Ishino, F. (2006). Deletion of Peg10, an imprinted gene acquired from a retrotransposon, causes early embryonic lethality. *Nature Genetics*, *38*(1), 101–106. doi:10.1038/ng1699

Pai, A. A., Bell, J. T., Marioni, J. C., Pritchard, J. K., & Gilad, Y. (2011). A Genome-Wide Study of DNA Methylation Patterns and Gene Expression Levels in Multiple Human and Chimpanzee Tissues. *PLoS Genet*, *7*(2), e1001316. doi:10.1371/journal.pgen.1001316

Payseur, B. A., & Nachman, M. W. (2005). The genomics of speciation: investigating the molecular correlates of X chromosome introgression across the hybrid zone between Mus domesticus and Mus musculus. *Biological Journal of the Linnean Society*, *84*(3), 523–534. doi:10.1111/j.1095-8312.2005.00453.x

Pedersen, I. S., Dervan, P. A., Broderick, D., Harrison, M., Miller, N., Delany, E., … McCann, A. (1999). Frequent Loss of Imprinting of PEG1/MEST in Invasive Breast Cancer. *Cancer Research*, *59*(21), 5449–5451.

Poplinski, A., Tüttelmann, F., Kanber, D., Horsthemke, B., & Gromoll, J. (2010). Idiopathic male infertility is strongly associated with aberrant methylation of MEST and IGF2/H19 ICR1. *International Journal of Andrology*, *33*(4), 642–649. doi:10.1111/j.1365-2605.2009.01000.x

Reule, M., Krause, R., Hemberger, M., & Fundele, R. (1998). Analysis of Peg1/Mest imprinting in the mouse. *Development Genes and Evolution*, *208*(3), 161–163. doi:10.1007/s004270050168

Shi, W., Lefebvre, L., Yu, Y., Otto, S., Krella, A., Orth, A., & Fundele, R. (2004). Loss-of-imprinting of Peg1 in mouse interspecies hybrids is correlated with altered growth. *genesis*, *39*(1), 65–72. doi:10.1002/gene.20027

Tilghman, S. M. (1999). The Sins of the Fathers and Mothers: Genomic Imprinting in Mammalian Development. *Cell*, *96*(2), 185–193. doi:10.1016/S0092-8674(00)80559-0

Vrana, P. B., Fossella, J. A., Matteson, P., del Rio, T., O'Neill, M. J., & Tilghman, S. M. (2000). Genetic and epigenetic incompatibilities underlie hybrid dysgenesis in Peromyscus. *Nature Genetics*, *25*(1), 120–124. doi:10.1038/75518

Vrana, P. B., Guan, X.-J., Ingram, R. S., & Tilghman, S. M. (1998). Genomic imprinting is disrupted in interspecific Peromyscus hybrids. *Nature Genetics*, *20*(4), 362–365. doi:10.1038/3833