Effects of Beta Amyloid on the DNA Methylation Status of an in Vitro Model of Alzheimer's Disease

Noor Taher

A Senior Thesis submitted in partial fulfillment of the requirements for graduation in the Honors Program Liberty University Spring 2013
Acceptance of Senior Honors Thesis

This Senior Honors Thesis is accepted in partial fulfillment of the requirements for graduation from the Honors Program of Liberty University.

______________________________
Gary Isaacs, Ph.D.
Thesis Chair

______________________________
Mark Blais, D.P.M.
Committee Member

______________________________
Harvey Hartman, Th.D.
Committee Member

______________________________
Brenda Ayres, Ph.D.
Honors Director

______________________________
Date
Abstract

Available evidence points toward an epigenetic process in Alzheimer’s disease. This thesis describes the research that was done to investigate changes in DNA methylation using an in vitro model of the disease. Although the results indicated no global changes in methylation levels after treating differentiated IMR-32 cells with beta amyloid, there were several regions of the genome that changed their methylation status. Gene ontology studies revealed that these regions are associated with neuronal differentiation and cell fate genes, thus providing a possible model for the contribution of beta amyloid to the development of Alzheimer’s disease. This study provides incentive to further study the epigenetic processes in Alzheimer’s disease and explore avenues to reverse such epigenetic changes.
Acknowledgements

I would first like to thank my parents, for without their many sacrifices I would not be where I am now. I am also grateful for Dr. Gary Isaacs and his mentorship. He presented me with a great opportunity to experience the joy of scientific investigation, and he showed great trust in my judgment. I learned many, many things from him that helped better equip me for a future in research. I would also like to thank Dr. Mark Blais for his mentorship and allowing me to help him in developing the Histology lab. That opportunity led to many others, one of which was getting to work with Dr. Isaacs on this research. I would also like to thank Dr. Harvey Hartman for his valuable input while I was writing this thesis. I am also grateful for Rebecca Garrett, Courtney McKenzie, and Matthew Baker who worked with me on this project. Finally, I would like to thank all of my friends at Liberty University for their support during my undergraduate years.
Effects of Beta Amyloid on the DNA Methylation Status of an in Vitro Model of Alzheimer’s Disease

Introduction

Statement of the Problem

The classic hallmarks of Alzheimer’s disease (AD) are the presence of neurofibrillary tangles (NFTs) and beta amyloid plaques. The terminal abnormal cellular conditions of this neurodegenerative disease are well researched; however, the genetic factors that may play a role in the development of the disease remain less clear. Moreover, the epigenetic factors that cells use to modulate the DNA sequence are even less clear. Although several lines of evidence suggest that epigenetic modifications, like DNA methylation, might play a role in Alzheimer’s disease, it remains to be determined if an epigenetic signature can be a telltale sign of AD development.

The identification of epigenetic marks associated with AD development would provide excellent diagnostic markers for mapping AD progression and propose an epigenetic model to how show AD initially begins. In addition, a non-mutational, reversible process like DNA methylation would be vulnerable to the development of epigenetic therapeutics aimed at preventing or even undoing age-related changes to the genome. This thesis represents a novel line of research that may lead to significant progress in our understanding of AD progression and the biology of aging.

Background

Epigenetics. The cytosine of the 5’-CpG-3’ dinucleotide serves as the target for DNA methylation. Approximately 40% of human promoters contain CpG rich domains that typically contain low levels of DNA methylation (1, 2). The presence or absence of
the methyl mark across the DNA landscape can regulate the binding of various transcription factors to the promoter regions. For instance, the transcription factor Ets-1 binds to its recognition sequence upstream of the Foxp3 gene promoter only when that sequence is devoid of DNA methylation. Because this sequence is normally methylated in most tissues, Ets-1 binding (and thus Foxp3 promoter activation) is typically inhibited (3). On the other hand, transcriptional repressors like MeCP2 require CpG methylation near promoter regions in order to bind DNA and ultimately repress transcription (1). Since CpG methylation can either prevent the binding of factors that promote transcription or enable the binding of transcriptional repressors, gene silencing is the major biological outcome of DNA methylation (4) although exceptions are sure to exist (5).

Unlike mutational changes in the genome, epigenetic marks (such as the post-translational modification of histones and the methylation of cytosines) can dynamically alter the local chromatin environment which results in dynamic changes in gene expression and genomic stability (see (6) for a review). Interestingly, changes in genomic methylation patterns are strongly associated with various disease states such as cancer and syndromes involving mental retardation (7). These disease-associated epigenetic changes produce an overall hypomethylated genome, although some portions of the genome have restricted regions of CpG hypermethylation (8, 9). Understanding where these specific epigenetic changes have occurred will not only highlight the genomic regions most likely responsible for the overall disease pathology, it will also provide useful targets for future therapies.
Alzheimer’s Disease. Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by the presence of neurofibrillary tangles (NFTs) and amyloid plaques. The production of these structures results, in part, by the accumulation of hyperphosphorylated tau (10-13) and Aβ peptide (14, 15), respectively. While normal tau proteins add stability to microtubule structures, hyperphosphorylated tau leads to microtubule disassembly and a breakdown of the neuronal cytoskeleton (11). Likewise, Aβ deposits can produce neuronal, endothelial and glial cell death through several apoptotic pathways (16, 17). Even though several of the aberrant cellular conditions of AD are well known, the initial genetic factors which contribute to the development of AD are unclear.

Gene expression studies have been conducted to determine some of the transcriptional changes that result from artificial Aβ peptide treatments. These studies were quite limited in their scope either because they were conducted in undifferentiated neuroblastoma cells (18) or because only a portion of the transcriptome (1,176 genes in mouse) was examined (19). Although more expression data are needed to accurately describe the status of AD cells, the underlying cause of these differences in promoter activity is even more important. This includes the epigenetic contributions, such as DNA methylation, which serve to modulate base-line DNA sequences in the neuronal cells.

Epigenetics and AD. Several lines of evidence suggest that epigenetic modifications, like DNA methylation, play a role in AD. First, late onset Alzheimer’s disease, which represents over 90% of AD cases, appears sporadic in nature with no known genetic cause (20). Consistent with this, the inheritance pattern of AD in twins suggests a non-Mendelian mode of acquiring the disease (21-23) indicating that DNA
sequence alone does not explain AD development. **Second**, evidence of a parent-of-origin effect has been described for AD identifying several genomic regions that are transmitted through the maternal line to affected individuals (24). The requirement of an environmental contribution (in this case being the maternal germ line) is consistent with an epigenetic mechanism (25). It is interesting to note that one of the most common mechanisms of parent-of-origin effects is “genomic imprinting” mediated by DNA methylation (26). **Third**, studies in mice and humans suggest a global change in DNA methylation when comparing AD subjects to their control counterparts. In humans, monozygotic twins discordant for AD displayed differential DNA methylation levels in the anterior temporal neocortex, a region severely affected in AD (27). The regions of the brain not affected by AD displayed identical DNA methylation levels demonstrating the specificity of this result. The specific reduction of DNA methylation in the AD twin may provide a rationale for the link between folate deficiency and AD, since one of the normal functions of folate is to donate methyl groups for DNA methylation reactions (28). **Finally**, the expression levels of several AD-associated genes (APP, β-APP cleaving enzyme, and neprylisin) are regulated based on the DNA methylation status of their respective promoters (29, 30). It is important to note that although AD is associated with a global hypomethylation of the genome, the neprylisin (31) promoter actually becomes hypermethylated in murine cerebral endothelial cells treated with Aβ (30). This evidence demonstrates that a gene can be affected by an epigenetic change that increases or decreases its normal methylated state. NEP (neprylisin) expression is significantly reduced in AD brains most likely due to methylation-dependent silencing (30, 32). Taken
together, these data strongly suggest that DNA methylation plays a key role in the
development of AD.

Although the information presented above provides a strong link between DNA
methylation and AD, the specific pattern of genomic methylation has yet to be
determined in an AD model system for all human promoters. Furthermore, it remains to
be determined if an epigenetic signature can be indicative of AD development and
possibly useful in the diagnosis and treatment of the disease. Revealing the specific
regions of epigenetic change will not only shed light on the molecular process of AD
development, but it will also indicate the regions most vulnerable to the development of
future AD therapeutics.

**Determining Methylated DNA Sequences**

Several methods have been described that detect the presence of methylated
dNA. The methods used for determining methylated CpGs will be discussed here.
Bisulfite sequencing (33) utilizes the chemical differences between methylated and
unmethylated cytosine. Sodium bisulfite reacts with normal cytosine (unmethylated) to
form a sulfonated cytosine that can easily be deaminated to form sulfonated uracil.
Following the removal of the sulfonate group by alkaline conditions, the template can be
used in a standard polymerase chain reaction (PCR) reaction using Taq polymerase.
Since Taq recognizes uracil as thymine, the resulting PCR product will have a thymine at
locations corresponding to unmethylated cytosines. Methylated cytosines, on the other
hand, are protected from the chemical transitions to uracil so no changes in PCR product
are detected (see (34) for review). Although reliable, this method is not easily adapted to
meet the requirements of a genomic-sized examination since sequencing at this scale is quite expensive.

A spin off of the bisulfite sequencing method utilizes PCR primers to discriminate between methylated and unmethylated templates (35). Primers are designed to overlap with the CpG region of interest. After the template is treated with sodium bisulfite to convert unprotected cytosines to uracil, methylation-specific primers are used to anneal to the cytosines that remain (due to their protection by methylation). A PCR product indicates the primer could anneal and reveals the methylation status. Like bisulfite sequencing, this method is also not easily adapted to a genomic-scale based on the time and expense associated with high throughput PCR.

The methylated DNA immunoprecipitation (MeDIP) method (36) uses an antibody to capture DNA fragments by way of their methylated cytosines. The capture of specific regions is expressed as a purification enrichment and can be determined using PCR or by hybridizing the material to genomic chip arrays. The latter platform allows the global determination of methylated regions. Because this method does not detect unmethylated cytosines, a failure to detect a CpG region of interest could be due to its hypomethylated state or a technique problem in the purification process.

Other methods of DNA methylation determination utilize the specificity of restriction endonucleases for a particular CpG methylation state (methylation-sensitive restriction enzyme; MSRE) followed by PCR analysis. An example of an MSRE is HhaI, which recognizes and cuts 5’-GCGC-3’ sequences only when they are unmethylated (37). PCR of the region will produce a visible product only when the included CpG is methylated and thus protected from enzymatic digestion. This assay requires additional
PCR controls to determine if failed PCR runs are due to poor primer design or if the region is indeed hypomethylated.

This shortcoming can be resolved when two MSREs, with different methylation sensitivities, are used. For example, HpaII and MspI are isoschizomers that both recognize 5’-CCGG-3’ sequences. HpaII digestion is blocked when these sequences are methylated, while MspI is insensitive to the methylation status of the recognition site. When these fragments are cohybridized to genomic microarrays, (described as HpaII tiny fragment enrichment by ligation-mediated PCR; or HELP assay) intergenomic and intragenomic comparisons concerning DNA methylation can be made (38). Because this method can detect methylated and unmethylated regions on a genomic scale, the HELP assay is the method of choice for this study.

Materials and Methods

Overview

Figure 1 outlines the methods used to obtain the results. Briefly, cultured IMR-32 human neuroblastoma cells were differentiated and subsequently treated with beta amyloid (Aβ). DNA from undifferentiated, differentiated, and Aβ-treated will be referred to as undiff DNA, diff DNA, and Aβ DNA throughout the rest of this thesis, respectively. DNA from each group was isolated, purified and then digested with either HpaII of MspI restriction endonucleases. The DNA was then purified again and ligated to two sets of adapters of known sequences. Ligation-mediated PCR was then used to amplify all samples. A final purification procedure was performed before the samples were sent to NimbleGen for fluorescent labeling and co-hybridization on promoter microarray. Raw data from the microarrays were processed with computer software to
Figure 1. **Experimental Overview.** DNA was isolated and purified from differentiated, undifferentiated and Aβ-treated cells and subsequently digested with either HpaII or MspI restriction endonucleases. The DNA was then ligated to two sets of adapters of known sequences. Ligation-mediated PCR was then used to amplify all samples. The samples were sent to NimbleGen for fluorescent labeling and co-hybridization on a promoter microarray. Raw data from the microarrays was processed with computer software to determine and graph HpaII/MspI ratios for each probe.
determine and graph HpaII/MspI ratios for each probe. Site-specific PCR was then
performed on several randomly chosen loci to validate the microarray data and establish
its biological significance. Regions of significant change in methylation were mapped to
nearby genes, which in turn were annotated to their corresponding cellular processes.

Cell Culture

Cholinergic IMR-32 human neuroblastoma cells (39) were grown and
differentiated according to the procedure outlined by Curran et al. (40). The cells were
depleted of fibroblasts through incubation with immunomagnetic anti-fibroblast
microbeads followed by separation with LD MACS columns, following the
recommendations of the manufacturer. The cells were maintained in culture in
proliferation medium (DMEM + glutamax, 5% FCS, 100 U/ml penicillin-streptomycin,
and 10 µg/ml gentamicin) at 37°C in an atmosphere of 5% CO₂. The cells were plated at
a density of 5 × 10⁵ cells/15 cm plate and grown for 48 hours in this proliferation media.
The proliferation media was then replaced by differentiation media (DMEM with
glutamax, 2% FCS, 2mM sodium butyrate, 100 U/ml penicillin-streptomycin, and 10
µg/ml gentamicin). The cells were maintained in differentiation media for 7 days, and
then treated with 25 µM Aβ₁₋₄₀ peptide prepared as described in Chen et al. (30) or
vehicle for 48 hours. The cells were then rinsed with cold PBS (phosphate-buffered
saline), harvested by scraping, pelleted by centrifugation in 1.5 ml Eppendorf tubes, and
stored at -80°C.

DNA Isolation and Purification

The undifferentiated, differentiated, and Aβ-treated cell pellets were thawed and
kept on ice until they were treated with 478 µl of stop buffer (20% SDS, 5M NaCl,
.5MEDTA), 20 µl protease K (2.5 mg/ml), and 2 µl RNase. The reagents were mixed with the pellets by gentle agitation and the tubes were incubated in a 42°C water bath overnight.

Each sample was treated with 500 µl of phenol-chloroform-isoamyl alcohol (PCIAA) and incubated on a rotary wheel for 10 minutes at 37°C. The samples were then separated into an organic and aqueous layer by centrifuging at 17,000×g for 5 minutes. The aqueous layer containing the DNA in each sample was transformed to new 1.5 ml Eppendorf tubes. This process was repeated once to ensure DNA purity.

To precipitate the DNA, 3× the sample volume in 100% ethanol was added along with 1/10 the sample volume in 3M sodium acetate, and .5 µl of glycogen (20 mg/ml) to each sample. The samples were then centrifuged at 17,000×g for 1 hour. The supernatant was removed and the pellets were subsequently washed gently with 70% ethanol. The pellets were finally resuspended in 200 µl of deionized water. The DNA in each sample was then quantified using UV spectroscopy.

**Digestion**

Each sample was digested with HpaII and MspI in separate reactions. Each reaction contained 1 µg of DNA and 4 µl of HpaII (10,000 U/ml) with 20 µl of New England Biolabs Buffer 1, or 2 µl of MspI (20,000 U/ml) with 20 µl of NEB 4 and. The reactions were brought to a final volume of 200 µl with deionized water and incubated overnight in 37°C. The DNA from each sample was then purified as described above and resuspended in 15.5 µl of 10 mM Tris-HCl (pH 8).
**Ligation**

To avoid degradation or re-annealing of the sticky ends, the ligation was performed immediately after the digestion. Two sets of adapters were used: NHpaII12 (5’-CGGCTTCCCTCG-3’), NHpaII24 (5’-GCAACTGTGCTATCCGAGGGAAGC-3’), JHpaII12 (5’-CGGCTGTTCATG-3’), JHpaII24 (5’-CGACGTCGACTATCCATGAACAGC-3’). The 24-mer and 12-mer oligonucleotides of each set were annealed together in preparation for the ligation. Ligation was performed on HpaII and MspI digested undiff DNA, diff DNA, and Aβ DNA. Reactions were carried out in PCR tubes and consisted of 6 µl of 5× T4 ligase buffer (250 mM Tris-HCl (pH 7.6), 50 mM MgCl₂, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000, Invitrogen), 15.5 µl digested DNA, 4 µl of 50 µM pre-annealed JHpaII linkers, 4 µl of 50 µM pre-annealed NHpaII linkers, and 1 µl of T4 DNA ligase (4 U/µl). The reactions were put in a thermocycler at 16°C overnight. The reactions were then diluted to 10 ml by 10 mM Tris-HCl (pH 8).

**LM-PCR**

The MspI digested DNA used in the amplification was half the volume of the HpaII digested DNA. This is due to the difference in complexity of fragments in the MspI digested DNA, which will cause these fragments to amplify and saturate the solution in the PCR faster and undergo “extra” cycles without amplification. This makes the sample susceptible to unwanted artifacts. This increased complexity in MspI fragments, and circumventing this issue by use half as much MspI digested DNA as HpaII digested DNA was previously reported(38). The LM-PCR reactions contained 5 µl of HpaII digested DNA or 2.5 µl of MspI digested DNA, .5 µl of JHpa 24-mer oligonucleotide, .5 µl of
NHpa 24-mer oligonucleotide, 25 µl of Supermix (Bio-Rad), and brought to a final volume of 50 µl with deionized water. The LM-PCR scheme began with an initial extension step at 72°C for 10 minutes, 15-20 cycles of annealing at 95°C for 30 seconds and 72°C for 3 minutes, and a final extension step at 72°C for 10 minutes.

**Purification**

The reactions were purified using Qiagen’s QIAquick PCR Purification Kit (50) according to the manufacturer’s instructions and DNA was quantified using UV spectroscopy.

**Microarray**

Five 385K DNA Methylation microarrays were purchased from Roche NimbleGen to hybridize 2 undiff DNA samples, 2 diff DNA, and 1 Aβ DNA. Following quality control requirements by Roche NimbleGen for human DNA, all samples were amplified to and purified to achieve a final mass of at least 4 µg of DNA and a concentration of at least 250 ng/µl.

**Data Analysis**

The raw microarray data from Roche NimbleGen was processed using Matlab 2011. First, correlation graphs were generated to examine the relationship between the 2 undiff DNA microarrays and between the 2 diff DNA microarrays. Each array contained 4,288 random probes designed by Roche NimbleGen for control purposes. The background fluorescence threshold was taken as 2.5 mean absolute deviations (MAD) above the median fluorescence signal of the random probes (41, 42). For a probe to be considered valid, both of its HpaII and MspI DNA signals have to be above the threshold, and in all 3 conditions (undiff DNA, diff DNA, and Aβ DNA). An HpaII/MspI ratio
histogram was generated for each condition (undifferentiated, differentiated, and Aβ-treated). The undifferentiated and differentiated data were an average of 2 microarray data sets for those conditions. The histograms were mean-centered and the x-axis was converted from linear scale to log$_2$.

**Site-specific Confirmations**

Site-specific qPCR confirmations were used to validate the microarray data and show their biological significance. Undiff, diff, or Aβ DNA was digested with HpaII and MspI in separate reactions as described above. An “uncut” control was included to serve as a positive control for maximum amplification possible. The recipe for the uncut digestion reaction was identical to that of MspI except that 2 µl of 50% glycerol were added instead of the enzyme. The samples were subsequently purified as previously described and resuspended in 200 µl of deionized water.

Initially, confirmations were aimed at exploring probes that exhibited significantly more or less HpaII/MspI ratio than the mean. Another focus of those confirmations was probes that showed the greatest changes in their HpaII/MspI ratio between diff and Aβ DNA. Primers were designed around the closest 5’-CCGG-3’ to such probes using Primer3 (http://frodo.wi.mit.edu/). The PCR product size range was chosen to be between 80-140 bp, the primer T$_m$ was 60±2, and the primer size was 20±2 bp. All other criteria were left as the default settings by the software. The 25 µl qPCR reactions contained .015-.025 µg of HpaII or MspI-digested DNA or uncut DNA, 12.5 µl Supermix, and forward and reverse primers with a final concentration of .625 µM each. Reactions lacking DNA template were also included as control for primer self-annealing and amplification. Amplification was performed using a BioRad MJ Mini Personal
Thermal Cycler. The touchdown qPCR scheme began with an initial melting step at 95°C for 5 minutes. The first cycle was repeated 19 times and consisted of a melting step at 94°C for 10 seconds, followed by annealing at 69°C for 30 seconds, and a final extension step at 72°C for 30 seconds. The annealing step decreased by .5°C each cycle. The second cycle followed immediately and consisted of a melting step at 94°C for 10 seconds, annealing at 59°C for 30 seconds, and then an extension step at 72°C for 30 seconds. This second cycle was repeated 24 times with a constant annealing temperature, and was followed by a final extension step at 72°C for 5 minutes. The amplification graphs were generated by BioRad CFX Manager 2.0. The quantification cycle (Cq) values were obtained by positioning the artificial horizontal line within the linear phase of amplification for all three curves - uncut, MspI, and HpaII DNA – for each primer pair (Figure 2).

**Figure 2. Determining Cq values.** The graph represents a typical graph generated at the end of a qPCR run. Each curve represents relative fluorescence readings from one well after each cycle. The artificial threshold line is positioned so that it is across the linear phase of all curves in question for a given primer pair. The point at which the threshold line intersects with a curve represents the Cq value for that curve.
Gene Ontology

Gene symbols were linked to the 222 most changing regions, 111 regions becoming hypomethylated and 111 regions becoming hypermethylated, by plugging the region names into GREAT, a region/gene association and annotation software developed by Stanford University and Bejarano Lab (43). Human genome 18 was used for the species assembly and the whole genome for the background regions. This software developed graphs representing the number of associated genes per region, distance to TSS, and absolute distance to TSS. Under the global controls options, “View all region-gene associations” was chosen to obtain all of the gene symbols for the inputted regions. These gene symbols were then plugged into GeneCodis (44-46), a gene annotation website; homosapiens was used as the organism and biological processes was selected for genome wide assessment. The gene symbols of the regions becoming more hypomethylated, more hypermethylated, a combined list, and a random list of 111 regions from the microchip data were assessed using the following G.O. requirements: level 7 (the most stringent), a minimum of 3 genes associated with that ontology, a chi square value that was lower than the lowest obtained chi square value from the random list. False discovery rate was used to find a corrected chi square value. The enrichment value from which the chi-square value was obtained was calculated by dividing the ratio of genes for a gene ontology in the list to the total number of genes in the list by the ratio of genes for that gene ontology in the genome to the total number of genes in genome.

\[
\frac{\left(\frac{\text{Number of Genes for G.O. in List}}{\text{Number of Genes in List}}\right)}{\left(\frac{\text{Total Number of Genes for G.O.}}{\text{Total Number of Genes in Genome}}\right)}
\]
The gene ontologies that met these requirements and were associated with the central nervous system were charted and linked with their gene symbols.

**Results**

**Global DNA Methylation Distribution in Neurons Remains Largely Unchanged after Aβ Treatment in Vitro**

IMR-32 human neuroblastoma cells were differentiated and subsequently treated with Aβ or vehicle for 48 hours. DNA from experimental and control cells was isolated, differentially digested with HpaII and MspI, amplified, and hybridized to a promoter microarray. Analysis of the microarray data revealed a bimodal frequency distribution of the fluorescent loci in the control sample, a distribution that remains largely unchanged in the Aβ treated sample (r=.9870) (Figure 3A and B). The bimodal distribution of the data is consistent with the expectation that CpG dinucleotides fall into hypomethylated or hypermethylated categories. The overall consistency of the distribution after Aβ treatment suggests that only a select few genetic loci – corresponding to certain cellular processes – are epigenetically affected by Aβ.

**Site-specific qPCR Confirms the Microarray Data**

Since DNA methylation decreases from left to right along the x-axis in Figure 3A and B, it is expected from a probe which has an HpaII/MspI ratio higher than the average of the red curve to have a nearby CCGG that is less methylated in comparison to a CCGG laying close to a probe with a ratio lower than the mean of the blue curve. To confirm the predictions of the microarray, regions (A “region” is a chromosomal range that encompasses a group of neighboring probes. These chromosomal ranges were specified by NimbleGen in the raw data output and serve as “parent” designations for the included
Figure 3. Global DNA Methylation Distribution in Neurons Remains Largely Unchanged After Aβ Treatment in Vitro. (A and B) The log$_2$ HpaII/MspI ratios for the control and Aβ-treated microarray probes are plotted as frequency distributions. The data are mean-centered at zero (vertical grey line), with negative ratios and positive ratios signifying loci more and less methylated than the mean level of methylation, respectively. The blue and red curves represent the two components of the bimodal distribution. Each curve shows the probability of the data of each bin belonging to the left peak or the right peak of these bimodal distributions. (A) The mean HpaII/MspI ratios of the blue and the red curve are -0.66 and 1.76, respectively. (B) The mean HpaII/MspI ratios of the blue and the red curve are -0.76 and 1.95, respectively.
probes) were randomly chosen from a list of candidates that have 2-5 probes with an 
HpaII/MspI ratio higher than the mean of the red curve or lower than the mean of the 
blue curve. In those regions, we used qPCR to target a CCGG that was (1) lose to the 
probe(s) which qualified the region for this analysis and (2) had an appropriate 
surrounding DNA complexity to facilitate specific primer design.

Figure 4A showcases a region that has two probes with ratios higher than the 
average of the red curve in the control sample frequency distribution (Figure 3A). For 
comparison, Figure 4B shows a region that has 4 probes with ratios lower than the 
average of the blue curve in the same frequency distribution. Accompanying qPCR 
graphs of the highlighted CCGGs show approximately 40 times difference in 
methylation. In other words, in the population of differentiated IMR-32 cells, the CCGG 
in Figure 4A is 40 times less methylated than the CCGG in Figure 4B.

We performed the same confirmation experiments on data from the Aβ 
microarray (Figure 5A and B). The chromosomal region in Figure 5A has 5 probes with 
HpaII/MspI ratios higher than the average of the red curve in Figure 3B. The region in 
Figure 5B has 3 probes with ratios lower than the average of the blue curve in Figure 3B. 
Looking at the two accompanying qPCR graphs, it is apparent that the CCGG in Figure 
5B is approximately 15 times more methylated than the CCGG in Figure 5A in the 
population of Aβ-treated neurons. These results demonstrate that the probe ratios are 
indicative of actual methylation levels of nearby CpGs. Therefore, biologically 
significant conclusions can be reliably obtained from the microarray data.
Figure 4. Site-specific qPCR Confirms the Microarray Data (Control Sample). (A and B) The landscapes of microarray regions selected for confirmations are plotted with purple bars representing the probes and blue circles showing the CCGG loci in those regions. The qPCR results for the chosen CCGGs (red circles) are generated by setting the $2^{-(Cq)}$ of the MspI-digested DNA qPCR curve to “1” and setting the HpaII bar to represent the value of $2^{-(Cq_{HpaII}-Cq_{MspI})}$. 
Figure 5. Site-specific qPCR Confirms the Microarray Data (Aβ Sample). (A and B) The landscapes of microarray regions selected for confirmations are plotted with purple bars representing the probes and blue circles showing the CCGG loci in those regions. The qPCR results for the chosen CCGGs (red circles) are generated by setting the $2^{-(C_q)}$ of the MspI-digested DNA qPCR curve to “1” and setting the HpaII bar to represent the value of $2^{-(C_{q_{HpaII}}-C_{q_{MspI}})}$. 
**Aβ Induces High Changes in Methylation Levels in a Subset of Loci**

Although Figure 3 showed that the global DNA methylation status of the control sample genome does not change after treating the cells with Aβ, specific loci may still undergo change in methylation levels in response to Aβ treatment. Subtracting the HpaII/MspI ratios of the control microarray from the Aβ microarray produces the normal distribution shown in Figure 6A. Probes from only 0.05% of either end of the normal distribution were used for further confirmations and gene ontology analysis (Figure 6B and C). This portion represents a group of probes that changed their signal the most in response to Aβ treatment. With such stringency, we are eventually ensuring the selection of the biological processes that are most affected by Aβ treatment.

**Aβ-induced Probe Ratio Changes Correspond to CpG Methylation Changes**

Figures 4 and 5 demonstrate that the control sample and Aβ sample microarrays are successful in predicting CpG methylation differences within each sample. However, before proceeding with gene ontology analysis, it was necessary to confirm that a ratio change of the same given probe between the control sample and the Aβ sample microarrays corresponds to a same-CpG change in methylation between the control DNA and Aβ-treated DNA. Probes were chosen randomly from the “upper” and “lower” 0.05% of the most changing probes and qPCR was performed on the upstream and downstream CCGGs flanking each chosen probe in differentiated DNA and Aβ-treated DNA. Figure 7A contains a landscape of a region with a probe that shows a significant increase in HpaII/MspI ratio, and Figure 7B shows a region with a probe that displays a significant decrease between the control sample and the Aβ-treated sample. The
Figure 6. Aβ Induces High Changes in Methylation Levels in a Subset of Loci. (A) The data from the control microarray are subtracted from the Aβ data, and the result is plotted as a frequency distribution. The two vertical lines represent the “upper” and “lower” 0.05% cutoff positions in respect to the rest of the distribution. (B) A close-up frequency distribution of the “lower” 0.05% of the probes with the highest decrease in HpaII/MspI ratio (C) A close-up frequency distribution of the “upper” 0.05% of the probes with the highest increase in HpaII/MspI ratio.
Figure 7. Aβ-induced Probe Ratio Changes Correspond to Methylation Changes at Nearby CpGs. (A and B) The landscapes of microarray regions selected for confirmations are plotted with purple bars representing the (Aβ-control) value for the probes and blue circles showing the CCGG loci in those regions. The “control” and “AB” bars in the qPCR graphs represent the value of $2^{\Delta \left( C_{HpaII} - C_{MspI} \right)}$ in the control and Aβ sample DNA, respectively.
accompanying qPCR graphs for each region demonstrate that the change in probe ratios are confirmed with parallel changes in CpG methylation levels after Aβ treatment.

**Aβ alters the DNA Methylation of Cell Fate Genes**

The low number of high changes in probe ratios as seen in Figure 6 suggests that Aβ alters only a handful of cellular processes epigenetically in vitro. To shed light on these cellular processes, we mapped the 222 chromosomal regions that contained the highly changing probes to nearby genes using GREAT online software. Some regions were as close as 70-300 bp away from the nearest genes, while other regions were more than 150,000 bp away (data not shown). The output gene symbols were then annotated using GeneCodis online software. GeneCodis also generated p-values indicating the likelihood of several genes belonging to the same gene ontology being in our list of annotated genes. The results showed high levels of enrichment for genes associated with neuron differentiation, neurogenesis, and apoptosis (Table 1). Although Aβ treatment does not change the global DNA methylation status of the genome, it seems to effectively target select loci that have high impact on the fate of the cells. The probability that a number of genes in one ontology would appear in our list together can be contrasted with the probability of a random number of genes and their ontologies appearing in a list the same size as ours (Table 1).

To shed light on the probe ratio changes in respect to the landscape of one of our significant genes, we plotted the TSS of DLX1 and the regions associated with it, including the most significant changing probe that mapped to DLX1 in GREAT (Figure 8). The five regions surrounding the TSS showed small ratio changes in both directions.
<table>
<thead>
<tr>
<th>Region List</th>
<th>Gene Ontology</th>
<th>P-value</th>
<th>Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genes Becoming Hypomethylated</td>
<td><strong>Neurogenesis</strong>&lt;br&gt;Gliogenesis&lt;br&gt;Oligodendrocyte Differentiation&lt;br&gt;Regulation of Neurogenesis&lt;br&gt;CNS Neuron Differentiation&lt;br&gt;Neuron Fate Commitment&lt;br&gt;Regulation of Glial Cell Differentiation&lt;br&gt;(DLX1,DLX2,NKX6-2)&lt;br&gt;Cerebral Cortex GABA-ergic&lt;br&gt;Interneuron Fate Commitment&lt;br&gt;(DLX1,DLX2)&lt;br&gt;<strong>Neurogenesis</strong>&lt;br&gt;CNS Development&lt;br&gt;Oligodendrocyte Differentiation&lt;br&gt;Gliogenesis&lt;br&gt;(DLX2,DLX1,SOX9,NKX6-2)&lt;br&gt;<strong>Regulation of Nervous System Development</strong>&lt;br&gt;(MBP,ACCN1,NKX6-2)&lt;br&gt;<strong>Negative Regulation of Cell Death</strong>&lt;br&gt;Negative Regulation of Apoptotic Process&lt;br&gt;(SOX9,VHL,HAND2)</td>
<td>&lt;.001</td>
<td>192</td>
</tr>
<tr>
<td>Genes Becoming Hypermethylated</td>
<td><strong>Brain Development</strong>&lt;br&gt;Forebrain Development&lt;br&gt;(PCNT,MAPK8IP3,SKI)&lt;br&gt;<strong>Reg. of Programmed Cell Death</strong>&lt;br&gt;Regulation of Apoptotic Process&lt;br&gt;(GAS6,LYST,ADAM8)&lt;br&gt;<strong>Neurogenesis</strong>&lt;br&gt;(DLX1,PCNT,MAPK8IP3,SKI)</td>
<td>1.68×10⁻¹⁶</td>
<td>29</td>
</tr>
<tr>
<td>Random List</td>
<td>None</td>
<td>&gt;9×10⁻⁷</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Table 1. Aβ alters the DNA Methylation of Cell Fate Genes.** Parent region names of the “upper” and “lower” 0.05% most changing probes after Aβ treatment were mapped to the closest known genes. The generated list of gene names was annotated using GeneCodis. The p-values in this table represent the likelihood of a number of genes belonging to the same ontology to be in the same list of genes. A list of random genes of the same size as our list was also entered in GeneCodis to generate p-values associated with the likelihood of random genes with random ontologies being in a list the same size as ours. For stringency purposes, all ontologies listed have a p-value smaller than the p-value of the random list except for the first two p-values which are high due to the fact that all the genes that belong to those ontologies in the genome are in our list. Enrichment was calculated using the compound fraction described in the Materials and Methods section.
The most significant probe lies in a region that is approximately 6000 bp downstream of the TSS, indicating a high loss of methylation at that region.

**Discussion**

Our data reveal that the global DNA methylation status of the genome does not change when differentiated IMR-32 cells are treated with Aβ in vitro. However, lack of a global change in DNA methylation does not rule out changes at specific loci. Indeed, we confirmed that Aβ induces change in DNA methylation at discrete loci which are associated with cell fate genes such as neuron differentiation and apoptosis.

When we observed a change in the HpaII/MspI ratio of a given probe, it was not possible to pinpoint with absolute certainty which CCGG around that probe was changing its methylation level and producing the change in the ratio. For that reason, we chose our
method of testing one downstream and one upstream CCGG of the probe of interest. It should be noted that it could be changes in methylation in farther CCGG that produce the changes in probe ratios. However our high rate of confirmation on the upstream and downstream CCGGs reveals the high likelihood that changes in DNA methylation by Aβ are not pinpoint processes, but when there is a methylation change at one CpG it is likely that neighboring CpGs follow.

After mapping the microarray regions that showed significant change in methylation to nearby genes, we found that some regions were 70-300 bp away from the nearest genes, while other regions were more than 150,000 bp away. Although some high changes in methylation are fairly distant from the TSS, it is well-established that gene regulation can proceed distally through enhancer mechanisms (for review see (47, 48)). Future studies can focus on those regions and evaluate their candidacy as possible distal enhancer regions. It is also important to note that GREAT software maps regions to the closest “canonical” TSS of a given gene. It is possible, then, that GREAT is ignoring potentially closer TSS sites. In other words, some of the regions that highly change their methylation status in our data may be closer to a nearby gene than the data may seem to suggest.

A previous study by Cobos et al. shed light on the involvement of DLX transcription factors on the migration and differentiation of GABAergic interneurons (49). Interestingly, they found that DLX1/2 double mutant mice have interneurons that fail to migrate from the subpallium to the cortex and show immature increase in neurite growth. These results demonstrated the importance of DLX1 and DLX2 in the repression of neurite growth and maintaining a compact cell shape that is necessary for easy
migration maneuvers. With these previous findings in mind, our data seems to suggest the involvement of $A\beta$ in “de-differentiating” neurons and the consequent loss of their specific functions. Such effect could lead to the memory loss and aberrant brain function accompanied by AD. We therefore encourage future research to directly examine the expression levels of DLX1 in AD brain tissue as that may be a key step in pathogenesis.

**Conclusion**

We can make three conclusions from the results presented herein. First, $A\beta$ treatment of differentiated neurons in vitro does not change the global DNA methylation levels of the genome. There is an overall consistency in the structure of the distribution of methylation levels before and after $A\beta$ treatment. There is also a high correlation in ratio values between the control sample probes and the $A\beta$ sample probes of the same loci. Second, exposure to $A\beta$ does lead to significant changes in methylation at a subset of loci. Although the majority of the loci show largely unchanged methylation levels, some loci show high changes in DNA methylation following $A\beta$ treatment. Third, these loci are associated with genes involved in neuronal differentiation, neurogenesis, and apoptosis control. Therefore, $A\beta$ seems to contribute to AD progression through propagating neuronal loss of function and death.
References


