

## **Introduction**

Cancer is a multifaceted disease arising from genetic and epigenetic perturbations. Since cancer is a complex and heterogeneous disease among patients, successful recovery requires personalized treatment strategies targeted at the genetic and epigenetic perturbations in that individual.<sup>1</sup>

Cancer research has elucidated many mechanisms of tumorigenesis. Currently a relatively new and highly active field of cancer development involving epigenetic modifications promises to provide important insights into tumorigenesis, biomarkers, and potential targets for treatment. Aberrant DNA methylation as a global genomic event in cancer was identified in the early 1980's, and great progress has been made in understanding the role of DNA methylation in normal development and tumorigenesis.

However there still exists a shocking lack of understanding of the regulatory mechanisms involved in DNA methylation and the enzymes that catalyze the reaction. This plan proposes to discover protein-protein interactions between the Dnmt3 methyltransferase family and regulatory or accessory proteins. It is hoped that identification of these proteins can lead to a fundamental understanding of regulation of *de novo* methylation, and by extension to tumor cell expression of aberrant methylation patterns.

## **Background**

### ***Brief Explanation of Epigenetics***

Epigenetics is broadly defined as “the study of mitotically and/or meiotically heritable changes in DNA sequence.” Currently three primary mechanisms are encompassed under the epigenetic umbrella: DNA methylation, Polycomb-trithorax group protein complexes, and histone modification.<sup>2</sup> Epigenetics connects the genotype to environmental factors and determines inheritable gene transcription patterns, and hence affects the phenotype.<sup>1</sup> Of primary concern for this experiment is DNA methylation.

### ***DNA Methylation Reaction, CpG Islands, and the Methyltransferases***

DNA methylation is a covalent modification that adds a methyl group to carbon 5 of the cytosine ring. DNA methylation is accomplished by a family of proteins named DNA methyltransferases, for their ability to transfer a methyl group from the donor S-adenosyl-L-methionine to the 5 position of a cytosine base.<sup>2</sup> (Figure 1)

DNA methylation occurs at dinucleotide sequences where a cytosine residue precedes a guanine residue, denoted CpG.<sup>3</sup> In human somatic cells methylated cytosine residues account for roughly 1% of all genomic bases, thus 70% to 80% of the CpG dinucleotides in the genome are methylated.<sup>2</sup> There are regions that are rich in CpG dinucleotides that are non-randomly distributed through the genome, known as CpG islands. Interestingly many CpG islands exist at the 5' end of many human genes, and are typically non-methylated.<sup>2,3</sup>

Currently, there are three identified and characterized families of DNA methyltransferases: Dnmt1, Dnmt2, and Dnmt3. Mammalian development experiments have elucidated some functions of the DNA methyltransferases. Dnmt1 was the first identified methyltransferase, and subsequently mapped to the 19<sup>th</sup> chromosome. The N-terminal domain of Dnmt1 contains multiple domains important for import into the

nuclei, co-ordination of replication and methylation during S-phase, and limited suppression of *de novo* methylation. Dnmt1 has been designated the 'maintenance methyltransferase' due to its specificity for hemi-methylated substrates. Dnmt2 was identified by comparative database searches, and is located on the 10<sup>th</sup> chromosome. No clear function has emerged for Dnmt2; disruption of *Dnmt2* gene yielded no effect on global methylation during development, or the ability to methylate newly inserted retroviruses. While Dnmt2 maintains the motifs of DNA methyltransferases it has never been shown to have methyltransferase by assay. It has been suggested that Dnmt2 role is involved with centromere functions.<sup>2</sup>

The final Dnmt3 family consists of two enzymes: Dnmt3a and Dnmt3b, located on the 2<sup>nd</sup> and 20<sup>th</sup> chromosome respectively. Their established function is for *de novo* methylation primarily during development.<sup>4</sup>

### ***Purpose of Methylation and its Role in Development***

DNA methylation hinders transcription factor binding and promotes a condensed heterochromatin structure; thus DNA methylation is a powerful inhibitory mechanism.<sup>5</sup> Repression of genes is accomplished via two mechanisms. The first is that methylation directly interferes with protein binding its cognate DNA sequence. The second is mechanistically opposite; proteins bind methylated DNA. Proteins belonging to the second class, such as MBD1, MBD2, MBD3, and MeCP2, have been implicated in DNA methylation dependent transcription repression. Yet retroviral sequences and inactive X chromosome genes are often silenced days before *de novo* methylation occurs. Since other cellular mechanisms of gene silencing exist, the need for methylation raises an important developmental and evolutionary question.<sup>2</sup>

DNA methylation offers a mechanism to permanently and stably silence genes. It is critical in establishment of regulation of gene expression, X-chromosome inactivation, genomic imprinting, chromatin remodeling, and silencing of endogenous retroviruses.<sup>2,5</sup> DNA methylation also prevents chromosomal instability by hypermethylation of repetitive sequences, and protects against gene disruption by deactivation of transposable elements.<sup>2</sup>

DNA methylation is a critical event in mammalian embryonic development. Experiments targeting the Dnmt3 family for non-functional mutations demonstrated the importance of methylation in cell differentiation and embryonic development. Homozygous mutants Dnmt3a<sup>(-/-)</sup> and Dnmt3b<sup>(-/-)</sup> ES cell lines did not differentiate and retained expression of the undifferentiated ES cellular marker Oct3/4. The mutants were also unable to methylate proviral DNA, and lacked all *de novo* methylation. Dnmt3a<sup>(-/-)</sup> mutant mice were runted and died at ~4 weeks old. Conversely Dnmt3b<sup>(-/-)</sup> mice were not viable and showed vast developmental abnormalities. Mutations of the Human Dnmt3b is involved in ICF syndrome, characterized by immune deficiencies, centromeric heterochromatin instability, and facial abnormalities.<sup>5</sup>

During normal development and differentiation, DNA methylation levels rapidly change in a specific concerted manner in which gene specific demethylation and *de novo* methylation occurs.<sup>6</sup> During this time, small percentages of significant CpG islands become methylated which causes silencing of associated promoters. For example, human and mouse *Mage* genes are methylated in adult somatic cells, however remain unmethylated in germ cells.<sup>2</sup> Importantly *Mage* genes are methylation-dependent silenced

in virtually all tissues, however are expressed in malignant tumors.<sup>2</sup> Thus DNA methylation is critical for normal development and also tumorigenesis.

### ***Epigenetics and Cancer***

DNA methylation is a well studied regulatory mechanism with direct implications in cancer. Approximately 40% - 50% of human gene promoters are located within or near CpG islands. As described above, typically these promoters are non-methylated or contain tissue differentiation specific methylation patterns for genes no longer expressed. However analysis of hundreds of cancer genes compiled by the Sanger Institute's Cancer Genome Project, revealed that 77% of their promoter regions contained CpG islands.<sup>1</sup> Thus demonstrating an important link between epigenetics and cancer. Tumor cells display certain epigenetic hallmarks which include silencing of tumor suppressor genes associated with promoter methylation and global genomic hypomethylation.<sup>7</sup>

### ***Region Specific Hypermethylation and Tumor Suppressor Genes***

A lack of DNA methylation could be a requirement for active transcription. Establishment of biochemical mechanisms underlying methylation dependent silencing of gene promoters has yet to be fully established; however, it is apparent that promoter methylation functions to stop gene expression. Only silenced alleles in imprinted genes and inactive X-chromosomes exhibit fully methylated promoters. Promoter hypermethylation can help indicate loss of function in typical familial cancer genes arising in sporadic cancer. While research has not fully elucidated whether methylation of promoters in tumor cells is a cause or effect of gene silencing, it nevertheless mediates a loss of function that is critical for tumorigenesis.<sup>8</sup> Hypermethylation of promoters affects genes involved in cell cycle, DNA repair, metabolism, cell-cell interaction, apoptosis, and angiogenesis. (Figure 2)<sup>3</sup>

Cancer genes contain methylation at normally nonmethylated CpG islands. A number of tumor suppressor genes are included in this list. Loss of tumor suppressor genes is a critical event in tumorigenesis. Many mechanisms such as mutations or deletions lead to loss of function of tumor suppressor genes.<sup>8</sup> Hypermethylation can also serve as a mechanism in the 'Two Hit Hypothesis' of cancer development.<sup>3</sup>

The p15<sup>INK4A</sup> gene is frequently hypermethylated in acute myelogenous leukemias and other hematological cancers. It has been shown that p73, a p53 related gene, is hypermethylated in lymphomas. For p15<sup>INK4A</sup> and p73 DNA methylation appears to be the primary method of their inactivation. Epigenetic mechanisms have recently been observed in p16<sup>INK4A</sup>, specifically loss of function of p16<sup>INK4A</sup> in colon cancer is only associated with promoter hypermethylation. p16<sup>INK4A</sup> is critical in the cyclinD-Rb pathway, thus a mechanism of cell cycle control which is disrupted in nearly all tumors can be attributed to epigenetic mechanisms.<sup>8</sup> Among other genes that are affected by hypermethylation of their promoters include *Rb*, *VHL*, *hMLH1*, *BRCA1*.

### ***Genomic Hypomethylation***

Studies have shown that DNA methylation and chromosomal integrity are linked.<sup>9</sup> DNA hypomethylation causes chromatin decondensation and chromosomal rearrangements.<sup>5</sup> 40% of CpG islands are found in repetitive segments, while hypermethylated in normal cells, these CpG islands are typically hypomethylated in

cancer cells. Hypomethylation at these sites is correlated with greater genome instability.<sup>1</sup> Studies using the DNA methylation inhibitor 5-azacitidine revealed bizarre chromosomal rearrangements. In studies conducted on mice ES cells, hypomethylation increased mutations at certain loci 10 fold, suggesting that methylation hinders aberrant recombination events.<sup>2</sup> Specifically DNA hypomethylation of the pericentromeric satellite regions 2 and 3 on chromosome 9 correlated with urothelial carcinomas of bladder, ureter or renal pelvis. DNA hypomethylation was also linked to loss of heterozygosity on chromosome 9 in urothelial carcinomas. This suggests that DNA hypomethylation induced chromosomal instability is an active participant in carcinogenesis.<sup>5</sup>

### ***Epigenetic Events Preclude Tumor Formation***

Recently the significance of epigenetic mechanisms in gene function loss has been demonstrated that epigenetic processes precede and are essential genetic drivers of tumor development.<sup>8</sup> Findings have hinted that aberrant epigenetic changes proceed aberrant genetic defects necessary for tumorigenesis. Evidence suggests that epigenetics interfaces with genetics by being the principle receiver of harmful environmental impacts.<sup>1</sup>

Recent analysis of specific cancers has shown that epigenetic events such as aberrant DNA methylation are often early events in carcinogenesis. In hepatocellular carcinomas associated with HBV or HCV infection, in precancerous cirrhosis tissue DNA methylation changes on chromosome 16 had occurred. A loss of heterozygosity on chromosome 16 had been frequently found in advanced metastasized hepatocellular carcinomas. *E-cadherin* is a cell-cell adhesion molecule in adheren junctions. Loss of *E-cadherin* supports loss of adhesiveness resulting in metastasis in tumor cells. The *E-cadherin* gene is located on chromosome 16 near the previously mentioned aberrant methylation patterns. A correlation between DNA hypermethylation and *E-cadherin* silencing is supported by the reinduction of expression following 5-azacytidine treatment. *E-cadherin* is considered a tumor suppressor gene and loss of expression a critical event in tumorigenesis.<sup>5</sup>

*Helicobacter pylori*, a cause of gastric carcinogenesis, is known to promote regional DNA hypermethylation.<sup>1,5</sup> In cervical intra-epithelial neoplasia, a precursor for squamous cell carcinoma of uterine cervix associated with HPV infection, Dnmt1 expression has been shown to be increased. Expression levels of Dnmt1 steadily increase from low-grade cervical intra-epithelial neoplasia to squamous cell carcinoma. DNA methylation changes have also been observed in lung carcinogenesis. Patients who smoke have higher rates of DNA hypermethylation in both non cancerous lung tissues and non-small cell lung cancers than those who have never smoked. Renal carcinogenesis also expresses changes in DNA methylation. In noncancerous renal tissue of patients with renal cell carcinoma exhibited greater levels of CpG island hypermethylation compared with patients free of renal cell carcinoma. Increasing levels of CpG island methylation correlates with infiltrating growth pattern, vascular involvement, and progressively higher histological grade renal cell carcinoma. Thus DNA methylation could also be a mechanism of tumorigenesis in renal cell carcinoma.<sup>5</sup>

### ***Use of Epigenetics as Biomarkers for Tumor Detection***

Epigenetic markers offer several advantages over genetic markers. Epigenetic methods can accept a lower degree of sample purity than genetic methods. Genetic tests for mutations, single nucleotide polymorphisms, or loss of heterozygosity in cancer cells can be masked by normal cells, while epigenetic tests offer specificity in heterogeneous samples. Epigenetic tests allow plasma, stool, sputum, urine, and other non-invasive samples for reliable detection of DNA methylation changes. DNA methylation patterns are tissue specific. This marker could be used to identify the tissue of origin in metastatic tumors. Currently there are several potential epigenetic biomarkers that have been characterized.<sup>1</sup>

A potential marker for human bladder cancer was discovered when a hypomethylated state of circulating peripheral blood cells correlated with the disease. Progressive DNA hypomethylation in the LINE-1 sequence parallels the progression of prostate cancer.<sup>12</sup> Thus epigenetic biomarkers could also be used in early cancer detection.<sup>1</sup> DNA methylation at the D17S5 locus is strongly correlated with poorer differentiation of lung adenocarcinomas.<sup>5</sup> Currently methylation profiles of cancers are being compiled and the selection of genes for screening panels are being created.<sup>1</sup>

While many potential biomarkers have been found, current DNA methylation analyzing techniques, such as Sequeenom or Illumina, are not suitable for clinical applications due to the need for complex machinery and technical sample preparation.<sup>1</sup>

### ***Interaction***

In some cancers a change in expression of the DNA methyltransferases Dnmt1, Dnmt3a, and Dnmt3b have effects on the epigenome.<sup>5</sup> However studies are needed to investigate the mechanisms by which Dnmt3a and Dnmt3b catalyze *de novo* methylation.<sup>4,9</sup> Currently proposed are two hypotheses by which DNA methylation occur. The first is the ‘methylation by default’ mechanism which characterizes methylation as an indiscriminate process. The default hypothesis suggests that DNA methylation occurs when the genome is accessible to Dnmt3b.<sup>2</sup>

The second hypothesis suggests that accessory or regulatory proteins are required for DNA methylation. Evidence for the ‘accessory’ mechanism initially came from plants; studies involving the loss of chromatin remodeling protein SNF2 have demonstrated that genomic methylation patterns were significantly affected. Specifically hypomethylation of repetitive elements and imprinted regions were observed. It was observed that a *Lsh*<sup>(-/-)</sup> mutant’s genome contained ~40-55% reduction of methylation.<sup>10</sup> Other evidence comes from a study that found that Dnmt1 forms a transcription repressive complex with Rb (tumor suppressor), E2F1 (sequence specific transcription activator), and HDAC1 (histone deacetylase). Thus, this study linked DNA methylation, histone deacetylase, a growth regulatory pathway, and sequence specific DNA binding.<sup>11</sup> Viral products can also form complexes with DNA methyltransferases. The HPV-16 E7 protein has been reported to directly bind Dnmt1 and stimulate enzymatic activity.<sup>5</sup> Further evidence that the polycomb protein EZH2 recruits Dnmt1 and Dnmt3b to the *MYT1* and *WNT1* loci, where gene silencing occurs by DNA methylation.<sup>12</sup>

## **Experiment**

### ***Validation***

As mentioned above, virtually nothing is known about the regulatory mechanisms of *de novo* DNA methylation. Evidence has suggested that the DNA methyltransferases are influenced by direct protein-protein interaction. If the *de novo* methyltransferase family Dnmt3 is regulated by proteins it would have immense implications in the fields of developmental biology, cancer biology, cancer treatment and detection. Discovery of regulatory proteins would have direct implication in tumorigenesis. Once discovery and identification of regulatory proteins were completed, comparative studies could be completed in tumor cells. If it is discovered that disruption of methyltransferase regulatory genes or proteins occurred in cancer, this could potentially represent a target for cancer therapy. Since epigenetic aberrations appear prior to tumor development and screening from noninvasive samples are possible, a correction treatment for aberrant epigenetic events could in theory prevent the furthering development of a tumor phenotype. Thus a discovery assay could provide multiple fronts for continuing research including potential cancer treatment.

### ***Methods***

Co-immunoprecipitation has many advantages for protein complex detection, namely high specificity, ease of procedure, and compatibility with downstream analysis methods.<sup>13</sup>

Since Dnmt3b exist in both the cytosol and nucleus, this gives two distinct points for possible Dnmt3b regulation. However Dnmt3b functions as a nuclear protein; for this study nuclear regulation will be examined. Nuclear extracts of mouse ES stem cells were prepared and examined at different stages of the cell cycle. An advantage of ES cells is that *de novo* methylation is highly active during embryonic development and as a model of normal Dnmt3b activity. Monoclonal antibodies against Dnmt3b were commercially available from Santa Cruz Biotechnology Inc (clone #:52A1018 ). Monoclonal antibodies were chosen to increase specificity and to help eliminate nonspecific binding observable when using polyclonal antibodies. To qualitatively validate correct binding, immunohistochemical staining will be performed to verify a nuclear staining pattern.

The oriented affinity method of immunoprecipitation was chosen for two reasons; to eliminate antibody heavy and light chain contamination, and to allow sterically unhindered antigen binding. The antibodies were crosslinked to a Protein G beads by DSS.<sup>14</sup> The nuclear extracts will first be washed in a protein G resin to clear any protein-Protein G binding prior to addition of the antibody resin. Then the precleared nuclear extracts will be immunoprecipitated and eluted by low pH for further analysis by gel electrophoresis.

A 2D SDS-Page gel electrophoresis will be performed to isolate individual proteins by isoelectric point and molecular weight. The polyacrylamide gel will be visualized by a Coomassie based method, Gelcode Blue Stain reagent (Thermo Scientific) for increased clarity. The protein bands will be excised from the gel, and analyzed by liquid chromatography-tandem mass spectrometry for identification. Tandem mass spectrometry will be chosen because SDS-Page is insufficient to totally resolve complexes. Tandem MS allows identification of multiple proteins per band allowing identification of complex protein samples.<sup>13</sup>

### ***Future Candidates for Research***

Assuming that regulatory proteins are found this presents with multiple targets for continuing research. Any regulatory proteins would need to be classified. Their active domains would need to be characterized, and their biochemical method of regulation examined. As mentioned before, Dnmt3b could be regulated at the cytosolic level. This could be an impetus for another discovery assay, perhaps leading to another set of protein characterization. Interaction of these two possible areas of regulation would need to be explored to determine the exact interplay. The expression patterns of the various regulators during development would need to be categorized. While a host of “basic science” (NIH’s verbiage) research could be completed, a comparative study using tumor cells could be assayed. Regulatory proteins could be examined for aberrant regulation, mutations, deletions, or any other tumorigenic mechanism. Possible treatment methods could be developed to reactivate silenced tumor suppressor, or correct aberrant methylation patterns. The mechanisms of DNA methyltransferase regulation are virtually unknown and this study could present countless possible future research opportunities.

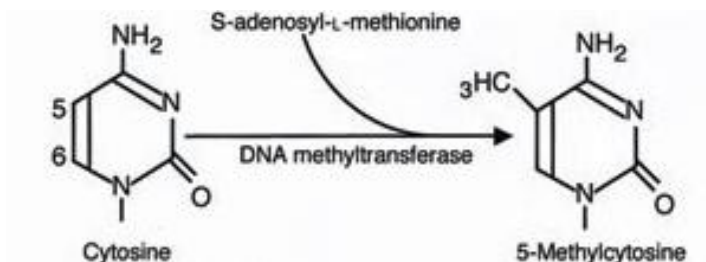


Figure 1: Methylation Reaction<sup>5</sup>

Tumor suppressor genes mutated in familial cancers	Other important genes
VHL	p15 <sup>INK4</sup>
p16 <sup>INK4</sup>	ER
E-cadherin	O <sup>6</sup> -MGMT
hMLH1	GS TP1
BRCA1	TIMP3
LKB1	DAFK1
	p73

Figure 2: Cancer Genes associated with Promoter Hypermethylation<sup>3</sup>

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