Targeting Epigenetic Processes in Glioblastoma Multiforme

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for Graduation with Distinction in Biological Sciences in the Undergraduate Colleges of the Ohio State University

By

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Abstract

Among tumors of the central nervous system, gliomas are the most prevalent and are associated with the poorest prognosis. Overall survival rates are low, and combined radio- and chemo-therapy provides only marginal benefit; thus it is essential to develop novel methods to treat patients with these aggressive tumors. One promising new area of research focuses upon epigenetics, the regulation of genetic expression without modifying the DNA base-pair sequence. Epigenetic mechanisms, such as histone acetylation, methylation, and cytosine methylation, have been shown to modulate tumor-suppressor genes in non-transformed cells. We hypothesized that epigenetic mechanisms contribute to the transformed phenotype of gliomas by targeting critical tumor suppressor genes. Our preliminary research showed that low doses (1 - 3 μM) of the histone deacetylase inhibitor HDAC-42 led glioma cell lines to undergo cell cycle arrest and apoptosis in a dose dependent fashion. Similar concentrations of HDAC-42 sensitized glioma cell lines to apoptosis induced by standard chemotherapeutic drugs (cisplatin, temozolomide). The diminished apoptotic threshold induced by HDAC-42 correlated with decreased sphingosine kinase-1 (SK1) activity, an important enzyme promoting survival in GBM tumors. Despite this promising result, the apoptotic effect was modest in several glioma cell lines, which led us to examine the importance of other enzymes that contribute towards tumor suppressor gene silencing. Recent work has demonstrated that HDAC enzymes associate with repressive SWI/SNF complexes containing other co-repressive elements to silence tumor suppressor genes. Among the epigenetic enzymes implicated in tumor-suppressor silencing is the protein arginine methyl transferase-5 (PRMT5), an enzyme that methylates histones H3 (H3R4) and H4 (H4R8) that localize to promoter regions of specific genes. Chromatin immunoprecipitation (ChIP) experiments have shown that PRMT5 directly targets the ST7 promoter, a known tumor suppressor gene. Western blot analysis of protein lysates isolated from 6 glioma cell lines has shown that PRMT5 is overexpressed, a finding that we have found to be critical for cellular transformation. Western blots have shown that ST7 not expressed in glioma lines but is abundantly expressed in non-transformed cells (B cells and fibroblasts). Through sequence-specific RNA interference, we are presently targeting PRMT5 expression and examining ST7 expression. These experiments will allow us to determine if re-expression of ST7 correlates

with restoration of features associated with control of cell growth and survival. Experimental therapeutic strategies to target multiple co-repressor enzyme activities are discussed.

Introduction

Gliomas are the most common primary brain tumor in adults (LeFranc, 2005). Although less than 2% of the new tumor cases in the United States annually are primary brain tumors, (Chamberlain) this relatively small incidence leads to disproportionate amount of mortality. The most prevalent form of glioma, glioblastoma multiforme (GBM), is also the most malignant (Collins, 2004).

Glial tumors form from astrocyte cells in the brain. Normal astrocytes assist neural function in several capacities, including the release of glucose to the extracellular space, the uptake of neurotransmitters, and the stimulation of oligodendrocytes to secrete myelin around the neurons (Piet, 2004). Several different tumors of varying morbidities can form from astrocytes, and their malignant potential is graded on a four-tiered system. More aggressive tumors will have (1) increased cellularity, meaning its cells will be more differentiated from normal tissue, (2) increased mitoses, or rapidly-dividing cells, (3) endothelial progression, or the growth of blood vessels within the tumor mass, and (4) necrosis, or the death of surrounding tissues (Collins, 2004). GBM possesses all four characteristics, leading to a WHO Grade IV ranking, the most aggressive.

Several treatment options are commonly pursued for treatment of GBM. Treatment often begins with surgical resection. In less aggressive tumors, surgical resection is often regarded as curative, whereas in the treatment of GBM, resection is only palliative. Resection pursues several goals. Surgery strives to balance the removal of as much tumor as possible with the maintenance of neurologic function. Because GBM is especially infiltrative and often multifocal (Devita, 2001), it is often impossible to remove the entire tumor mass. Consequently, in the case of GBM, resection cannot cure the disease, but does relieve symptoms through reduction of intracranial pressure and tumor burden. Surgery often provides a modest increase in median survival time by a few months.

Radiation treatment can also provide palliative benefit to GBM patients. However, the invasiveness of the disease again presents difficulties, as the radiation beam is focused on areas including both solid tumor tissue and the surrounding invasive tumor within normal tissue (DeVita, 2001). Acute toxicities include nausea, vomiting and headache, while later reactions include neurologic deficits due to radiation-induced demyelination of neurons or capillary permeability changes (DeVita, 2001). Learning capacity and abstraction ability can be impaired by radiation treatment. In severe cases, necrosis may occur.

Chemotherapeutic agents are often used, as well. The basic goal of chemotherapy treatment is to kill tumor cells with a sufficient specificity to minimize toxicity of normal cells. Significantly, tumor cells generally undergo rapid cell division, whereas most normal cells in adults are senescent. Because dividing cells must replicate their DNA, hindering DNA replication in theory serves as a desirable target for treatment. Consequently, agents such as carmustine, temozolomide, and cisplatin, which damage DNA through various chemical processes, are often used (Rabik, 2006). However, the specificity of these drugs is not absolute, as the tissues that still undergo cell division and growth, such as hair follicles, gastrointestinal epithelium, and reproductive organs, are adversely affected. Moreover, the restrictiveness of the blood-brain barrier prevents many otherwise-promising agents, such as bleomycin, doxorubicin, cisplatin, and vincristine, from reaching tumor in effective amounts at levels that would not cause dose-limiting toxicities (DeVita, 2001). Moreover, GBM tumors often evolve to become resistant to treatment with chemotherapeutic agents. The effect of DNA alkylating agents, for instance, can be mitigated by alkyltransferases in tumor cells that repair the DNA damage, allowing the tumor cells to survive treatment (Rabik, 2006).

While these standard treatments do provide benefit to GBM patients, their effects are modest. Combination radio- and chemo-therapy treatments improve median 50% survival times to approximately 55 weeks (DeVita, 2001). Because standard treatments provide patients with only modest benefit and substantial toxicity, it is necessary to explore other types of treatment strategies. Drugs that target epigenetic processes are currently being explored in early phase

clinical trials and preliminary data suggests that several of these agents hold great promise for patients with cancer.

All of the genetic information in a cell is encoded in its DNA, a long chemical strand composed of different nucleotides. These nucleotides act as the chemical letters that spell out the genetic information. Enzymes read this genetic information and transcribe into a messenger molecule, mRNA. This mRNA molecule is then translated through other cellular machinery known as ribosomes into proteins. These proteins can then perform specific chemical functions, such as breaking down sugars for energy, sending or receiving signals, and building cellular structures (Cambell, 2002). The individual genetic instructions (sequence) within the DNA that codes for a specific protein is known as a gene. Genes themselves are divided into several parts. The most important are the open reading frame that contains the information for the protein, and the promoter, a region usually immediately before the open reading frame. Regulatory proteins can bind to the promoters of genes, and depending on the type of regulatory protein, they can induce or inhibit transcription of the gene. (Snyder, 2003) Thus a cell can control when proteins are produced through promoter regions.

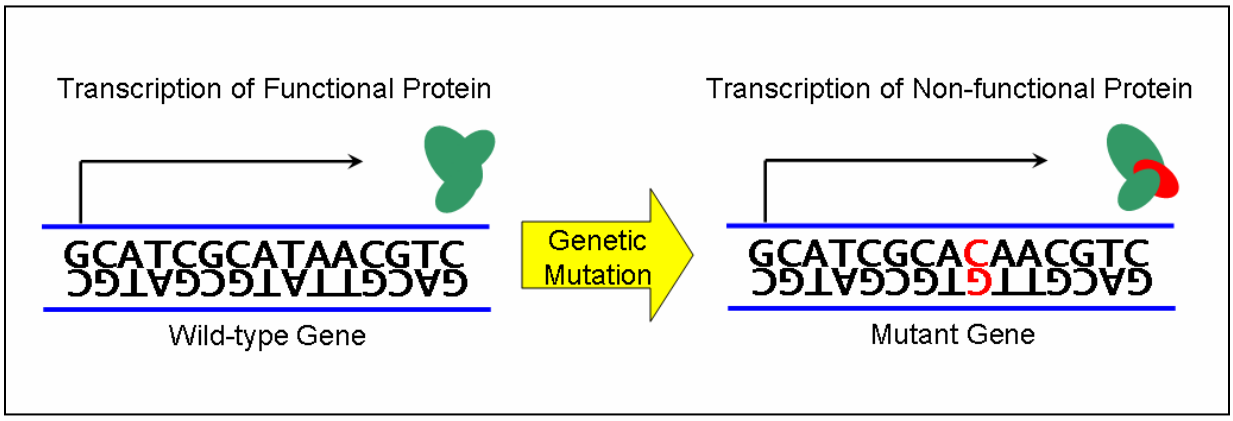

Figure 1. Genetic mutations can cause changes in protein structure. DNA replication errors and DNA-damaging agents, such as mutagenic chemicals and ultraviolet radiation, can change the base-pair sequence of genes. When these genes are translated, the resulting proteins may have a mutated amino acid sequence, possibly resulting in different structures and functions.

Conventional genetic events occur when the base-pair sequence of a gene is modified, potentially changing the information encoded. For instance, point mutations occur when a single nucleotide changed, perhaps due to the incorporation of an incorrect nucleotide during replication, or a chemical modification of the nucleotide due to UV irradiation or chemical mutagens. Alternatively, frame-shift errors can insert or remove a few nucleotides, leading to an erroneous reading frame during translation. Larger scale changes include deletions, duplications, or inversions of large sequences of nucleotides, or even chromosome translocations. (Kramer, 2002). These genetic modifications that affect the DNA code sequence can change the abundance and functionality of the proteins encoded by the gene. If the genetic changes affect the coded portion of the gene, the protein encoded by the gene affected can be changed (mutated). These changes can affect the protein's ability to bind with its substrate or with other regulatory molecules, causing it to work incorrectly. Alternatively, if the promoter region of the gene is changed, the protein can be produced under inappropriate circumstances (dysregulation), or over / under expressed.

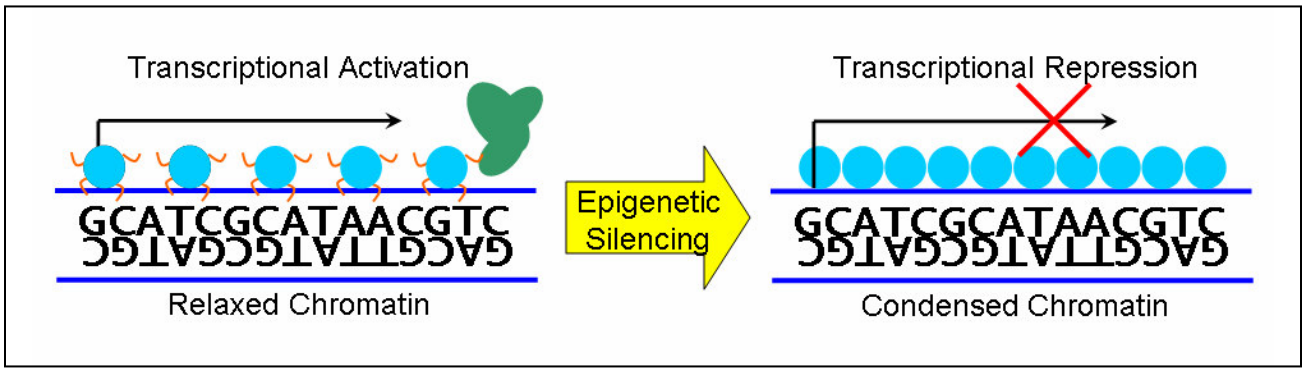

Figure 2. Epigenetic modifications can silence genes. By increasing the affinity of histones for the DNA strands, repressive epigenetic events can prevent the transcriptional enzymes from accessing the DNA strand. Transcription of the gene is then epigenetically silenced.

In contrast, epigenetic modifications do not change the base-pair sequence of a gene, and the proteins produced have the same sequences as before. Rather, epigenetic modifications acts as a switch to turn genes on or off. This switching mechanism occurs through the opening and closing of the DNA/protein superstructure referred to as chromatin (Jones, 2007). DNA associates with proteins known as histones in an arrangement similar to spaghetti wrapped

around a meatball. This provides the cell with an additional means of organizing its genetic program and provide an important layer allowing for tight control of gene expression regulation.

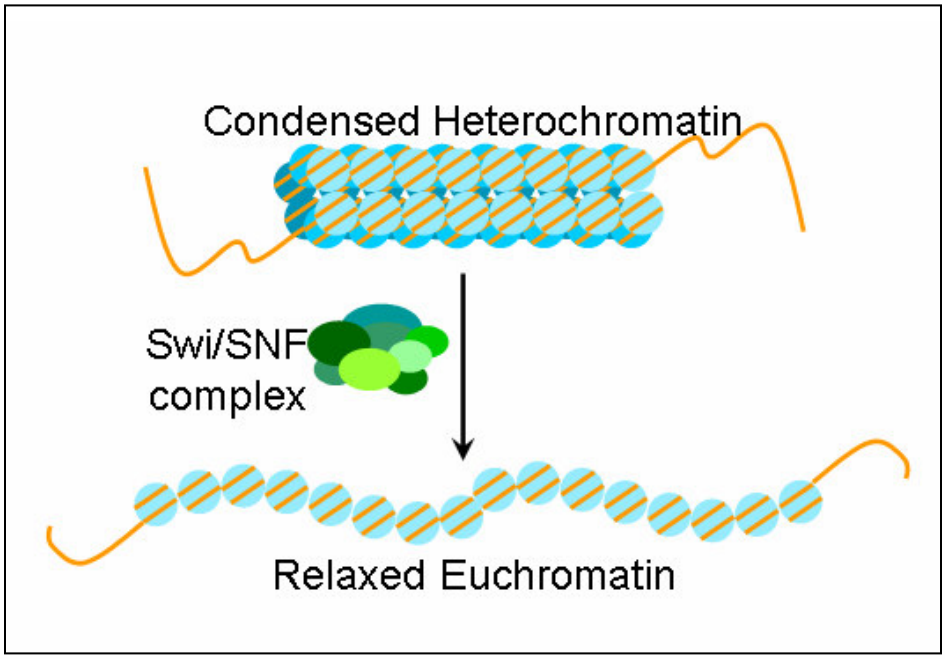

Figure 3. SWI/SNF modifies the chromatin superstructure. Chromatin remodeling complexes such as SWI/SNF can modify the chromatin superstructure, either converting condense heterochromatin to relaxed euchromatin (as shown) or reverting the relaxed euchromatin back to condensed heterochromatin. When the genes are condensed into heterochromatin, transcription is inhibited, but relaxed euchromatin allows transcription.

By default, chromatin structure is maintained in a compact/closed fashion that is not permissive to gene transcription. Specific covalent modifications to DNA and histones can alter the affinity a histone protein has for DNA and cause chromatin to assume a more open structure. Open chromatin allows chromatin remodeling complexes to further modify the protein/DNA structure leading to further "relaxation" of key promoter regions that, in turn, become more permissive to the docking of transcriptional machinery and expression of the protein of interest (Sif, 2004). The modulation of chromatin binding stems from the concerted effects of a two-step process. First, covalent modifications to the histones can change their affinity for the DNA. Second, chromatin remodeling enzymes change the superstructure of the chromatin according to the binding dictated by histone modifications. For instance, if histones bind DNA loosely, remodellers unwind DNA from histones, allowing transcription enzymes to

read the gene. Alternatively, if histone/DNA modifications result in a stronger affinity for DNA (DNA methylation, histone deacetylation, histone hypomethylation), chromatin remodelers will work oppositely, winding the DNA back up, and preventing transcription (Roberts, 2004).

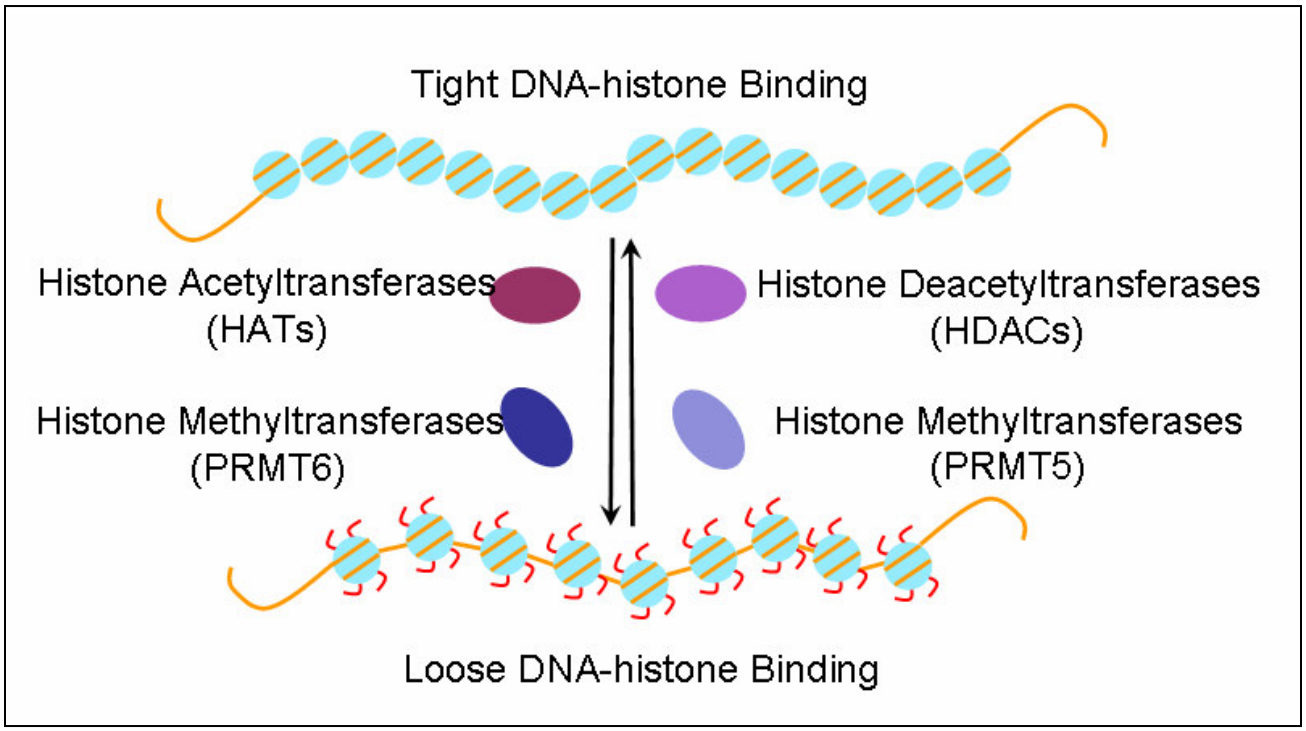

Figure 4. Histone-modifying enzymes modulate histone-DNA binding affinities. Histone Acetyltransferases (HATs) catalyze acetylation of N-terminal tails of histone proteins generally facilitates loose histone0DNA binding, which allows chromatin remodellers to reconfigure the chromatin superstructure and allow transcription. Histone Deacetyltransferases (HDACs) reverse this process. Certain specific histone methyltranserases such as PRMT5 facilitate loose histone-DNA binding and allow transcription, while other specific histone methyltransferases, such as PRMT6, facilitate tight histone-DNA binding and inhibit transcription.

The first step of altering DNA-histone affinities is affected by numerous modifications to proteins associated with the DNA. These changes are primarily achieved through covalent modification of histones. The N-terminal tails of the histone proteins serve as substrates for numerous difference chemical modifications, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and deimination, but the best characterized are acetylation and methylation (Kouzarides, 2007). Acetylation of lysine amino acids in the histone tails almost universally leads to transcriptional activation. The acetyl group neutralizes the lysines' positive charge, reducing its affinity for the negatively-charged DNA strand. Thus,

increasing the acetylation of histones causes a loose histone-DNA binding affinity, allowing chromatin remodellers to reshape the DNA, facilitating transcription. Histone tail acetylation is controlled by the effects of two opposing classes of enzymes, histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs), and changes in the relative activities of these enzymes can affect gene transcription (Bolden, 2006).

Another common modification to histone tails is methylation, which can occur on lysine and arginine residues. Methylation of lysine residues is caused by lysine methylases and reversed by lysine demethylases, while arnigines are methylated by argnine methylases. There are no known enzymes with arginine demethylating activity, although the methyl-arginine residue can be irreversibly degraded through deiminases (Kouzarides, 2007). The effect of these modifications is site-specific, as methylation on certain residues activates transcription, while other residues leads to gene silencing, and it is unclear exactly how these differing effects are mediated. For example, the protein arginine methyltransferase 5 enzyme acts to silence target genes by transferring a methyl group to the 8^{th} arginine residue on histone 3 (H3 R8) and the third arginine residue on histone 4 (H4 R3) (Pal, 2004).

Epigenetic dysregulation of important genes may play a crucial role in cancer. Normal cells express tumor-suppressor genes that check for defects in the cell and respond, often by preventing cell reproduction via induction of cell death or cell cycle arrest. Work in numerous tumors has found that tumor-suppressor genes are silenced genetic and epigenetic mechanisms. Genome-wide screens in GBM cell lines has shown that numerous genes are silenced epigenetically due to DNA methylation, including BIK, a pro-apoptotic protein (Kim, 2006). Treatment of GBM lines with SAHA, a histone deacetylase inhibitor, resulted in induction of p21WAF1 and P27KIP1, two genes that regulate cell cycle progression (Yin, 2007). Pal et al. has recently shown that PRMT5 associates with the chromatin remodeling complex SWI/SNF to turn off the tumor suppressor genes ST7 and NM23 (Pal, 2004). Our lab, in collaboration with Dr. Said Sif, has recently shown that PRMT5 works in concert with HDAC2 to repress ST7 expression in mantle cell lymphoma (Unpublished data). Thus, it is becoming clear that enzymes that covalently alter the chemical nature of chromatin are

capable of altering the cells threshold for transformation. Thus, it stands to reason that these enzymes, if dysregulated in human cancer, may serve as effective targets for anti-neoplastic therapy.

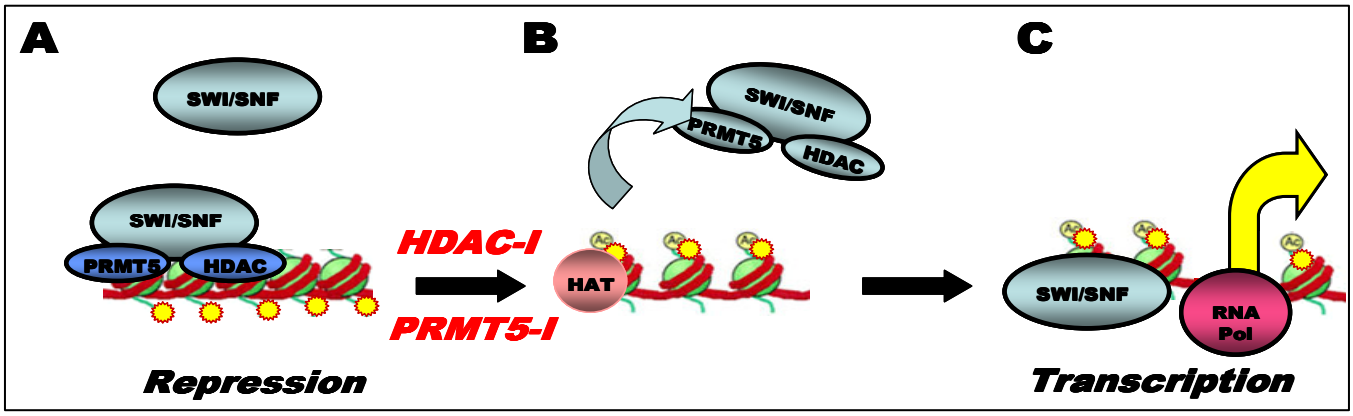

Figure 5. Epigenetic repression of anticancer genes and rationale for targeting HDAC and PRMT5 co-repressor enzymes in lymphoma. (A) Both activating and repressive SWI/SNF complexes co-exist in a the cellular proteome. Hypoacetylation of histones H3 and H4 promote PRMT5-driven methylation of arginine residues resulting in condensed nucleosomal structure and repression of target tumor suppressor genes. **(B)** Following treatment with drugs that inhibit HDAC (HDAC-I) and PRMT5 (PRMT5-I) activity, HAT enzymes are able to covalently modify N-terminal lysine residues on H3 and H4 histone subunits resulting in loss of ability for PRMT5 to maintain methylation of its target AA residues. Consequently, repressive SWI/SNF complex affinity for the modified chromatin is lost and these complexes become displaced. HAT and type I histone methyltransferases act to further modify nucleosome conformation. **(C)** Differential recruitment of activating SWI/SNF complexes remodel chromatin allowing for transcriptional machinery to gain access to regulatory regions on genomic DNA and for gene transcription of tumor suppressor genes to proceed.

HYPOTHESIS: A critical event that occurs during cellular transformation involves silencing of key cellular genes that regulate cell growth and survival. Enzymes such as PRMT5, HDACs and DNMT, have been shown to associate with repressive SWI/SNF complexes that directly target and silence several important regulatory genes. These three enzymes modify epigenetic events and act in concert to repress transcription of tumor suppressor genes like ST7. **Inhibition of these repressive enzyme activities will reverse silencing of tumor suppressor genes, mitigate cell cycle defects, reduce growth rate, and lower the apoptosis threshold of transformed GBM cells.**

Materials and Methods

Cell culture:U87MG, U251MG,A172, U118, U1242, U118, and M059K cell lines were kindly donated by James Van Brocklyn (Ohio State University). Gli36 cell line was kindly donated by Antonia Chiocca (Ohio State University). U87MG, U251MG, A172, U118, U1242, U118, and M059K lines were grown in Eagle's minimal essential medium (Gibco) with 10% fetal bovine serum (Gibco), non-essential amino acids, sodium pyruvate, penicillin (100 U/ml) streptomycin (100 ug/ml), and amphotericin B (0.25 ug/ml) (Gibco). Gli36 cells were grown in RPMI 1640 medium with 10% fetal bovine serum, penicillin (100 U/ml) streptomycin (100 ug/ml), and amphotericin B (0.25 ug/ml) (Gibco). Cells were cultured at 37℃ in 95% air/5% CO_2.

Drugs and reagents: HDACi 42 was obtained from Ching-Shih Chen (Ohio State University and stored in 10 mM stock solutions in DMSO at -80℃. Cisplatin was purchased from Bristol Myers-Squibb and was stored in 3.3 mM in PBS at -80℃. Depsipeptide was obtained from Fusijawa Pharmaceutical and was stored as a stock solution at 5 uM in acetonitrile at 4℃.

Western blotting: Samples were washed twice with cold PBS, before extraction with RIPA lysis buffer (150 mM NaCl, 1.0% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 5mM EDTA and 10 mM Tris, pH 7.4). Three times, samples were vortexed and incubated on ice for 10 minutes. Samples were then centrifuged for 10 minutes at 4℃ at 16,000g. Supernatants were the quantified using BCA assay with BSA standards. Samples were eluted in 6x Laemmli Buffer, and 30 ug of total protein was loaded in 10-12% SDS-PAGE gels, run at 90V for approximately 2 hours, and transferred to nitrocellulose membranes. Samples were blocked with 5% milk in 0.5% Tween 20-TBS. Membrane preparations were incubated overnight at 4℃ the appropriate primary antibody, then rinsed in 0.5% Tween 20-TBS, and incubated at 4℃ with the appropriate seconday antibody. Blots were visualized using the SuperSignal West Pico Chemiluniescent Substrate or SuperSignal West Femto Maximum Sensitivity Substrate (both Pierce), and exposing to Hyblot CL autoradiography film (Denville Scientific)

Antibodies used were rabbit-anti-PRMT5 (kindly donated by Said Sif, Ohio State University) at 1:500 in 5% milk-0.5% Tween 20-TBS, rabbit-anti-ST7 (kindly donated by Said Sif, Ohio State

University) at 1:400 in 5% milk-0.5% Tween 20-TBS, goat-anti-actin polyclonal IgG (Santa Cruz Biotechnologies) at 1:1000 in 5% milk-0.5% Tween 20-TBS, donket-anti-goat-HRP IgG (Santa Cruz Biotechnologies) at 1:3000 in 5% milk-0.5% Tween 20-TBS, and anti-rabbit-HRP F(ab')2 fragment from donkey (GE Healthcare, NA9340) at 1:3000 in 5% milk-0.5% Tween 20-TBS.

RNA interference: A DNA fragment with sequence as described by Pal et al (2004) was obtained from Said Sif. This DNA fragment was used as a template for an siRNA oligonucleotide targeting PRMT5 mRNA using the Ambion Silencer siRNA Construction Kit, using manufacturer's instructions. siRNA was quantified using the NanoDrop ND-1000, aliquoted in TE buffer, and stored at -20℃. Negative control siRNA sequence with no homology to any known open reading frame was obtained from MWG (Non-specific control dublex, 47% GC content).

siRNA transfection experiments were performed by seeding 6-well plates with either 5x104 U251MG cells or 1.5x105 U87MG cells in normal growth media, without antibiotics. Cells were grown until 30-50% confluent, then, siRNA at final concentrations from 2.5 to 40 nM was transfected using the Lipofectamine 2000 (Invitrogen) protocol, scaled to 6 well plates.

Flow cytometry: Cell cycle analysis was performed by first mildly trypsinizing cells.

For cell cycle analysis, Gli36 cells were stained with propidium iodide according to manufacturer's protocols, and flow cytometry was performed as described in Roychowdhury et al (2004).

For apoptosis staining, U87MG, Gli 36, and U251MG cells mildly trypsinized, washed twice in cold PBS, resuspended in Annexin Bindng Buffer (BD Pharmingen) at $1x10^6$ cells/ml. To $1x10^5$ resuspended cells, 5 ul of Annexin V-FITC (BD Pharmingen) and 5 ul of TOPRO 3 (Molecular Probes) were added, and the cells were incubated at room temperature protected from light for 15 minutes. Flow cytometry was performed according to manufacturer's protocols using BD FACScalibur (BD Pharmingen). The data were analyzed using WinMDI, using forward- and side-scatter to gate desired cell population, and plotting population's FL-4 fluorescence versus FL-1 fluorescence. Double-negative cells were regarded as living,

annexin-positive cells were regarded as apoptotic, and double-positive cells were regarded as dead.

Results and Discussion

HDAC inhibitors induce cell cycle arrest.

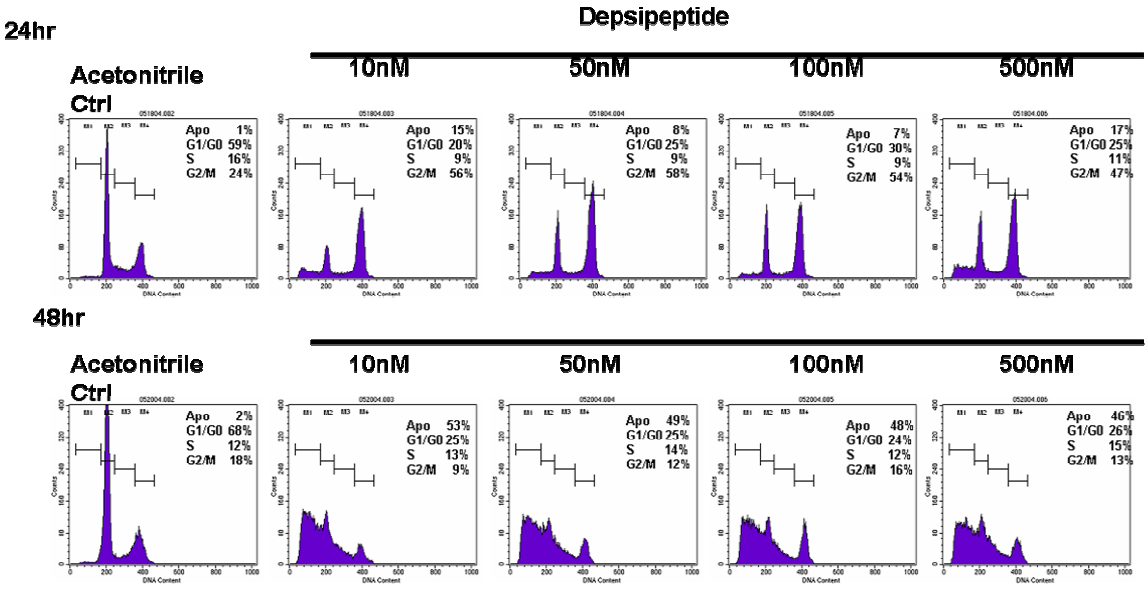

Figure 6. HDAC inhibitors induce cell cycle arrest. Cells were grown to 50% confluence and cultured in media containing varying concentrations of HDAC inhibitors. Cells were harvested at 24 and 48 hrs and stained with the nuclear dye propidium iodide. DNA content was then evaluated via flow cytometry. Cell cycle arrest was induced by depsipeptide at 24 hours, and DNA fragmentation was observed at 48 hours.

Gli36 cells were treated with either depsipeptide, a cyclical peptide inhibitor of HDAC currently undergoing Phase II trials or an acetonitrile vehicle control. As cells progress through the cell cycle, a series of steps required to divide, they must replicate all of their DNA. Measurement of the relative amounts of DNA within a cell provides an accepted measure of where cells are in the cell cycle. This is done through propidium iodide staining and flow cytometry. Cells are treated with with antibodies or dyes that adhere to a structure of interest. Antibodies are conjugated to fluorochromes, and the dyes often are themselves fluorochromes, so they emit a light of a given wavelength when undergoing excitation under a laser. By running prepared cells through a flow cytometer, the relative abundance of fluorescence within those cells can be measured. Propidium iodide is a stain that intercalates between the base pairs of DNA, so increased DNA content in a cell causes increased fluorescence. This assay produced a histogram output with an absolute cell count plotted against propidium fluorescence, or relative DNA content.

Within the 24 hour data, a comparison between the Depsipeptide-treated cells and the control indicates cell cycle arrest is occurring at the G2/M checkpoint (the point with the greatest amount of DNA). The peak at 200 fluorescence units represents cells in G1 phase, which have one copy of each DNA strand. As cells synthesize more DNA, the fluorescence increases, until a second peak is reached at 400 units, when the cells have two copies. In all of the depsipeptide-treated samples at 24 hours, a second peak accumulates at 400 units, while the second peak with duplicated DNA does not accumulate in the control. Consequently, we can conclude that cell cycle arrest is induced by depsipeptide at the G2/M checkpoint at 24 hours. In other work with mantle cell lymphoma cells, we have shown that HDACi causes induction / re expression of several cell cycle inhibitors that are found to be shut down in transformed cells.

At 48 hours, in depsipeptide-treated cells, fluorescence is observed below 200 units, indicating some cells have hypodiploid levels of DNA. This is indicative of apoptosis, the process by which cells undergo programmed cell death, as apoptotic cells degrade their DNA, resulting in cells with less than normal levels of intact genomic DNA. This phenomenon did not occur at 48 hours with only acetonitrile control, suggesting apoptosis was induced by the depsipeptide compound. Because an HDAC inhibitor appeared to induce apoptosis from this assay, we decided to test for this specifically, using a novel HDAC inhibitor, HDACi 42 developed at Ohio State University by Ching-Shih Chen, that has been previously shown to have anticancer properties in glioblastoma multiforme cell lines (Chen, 2005). Death can occur via apoptosis or by necrosis. Propidium iodide is not capable of distinguishing between the two mechanisms of cell death, therefore we performed a separate set of experiments looking for the detection of annexin V on the surface of cells treated with HDAC-I.

HDAC inhibitors induce apoptosis.

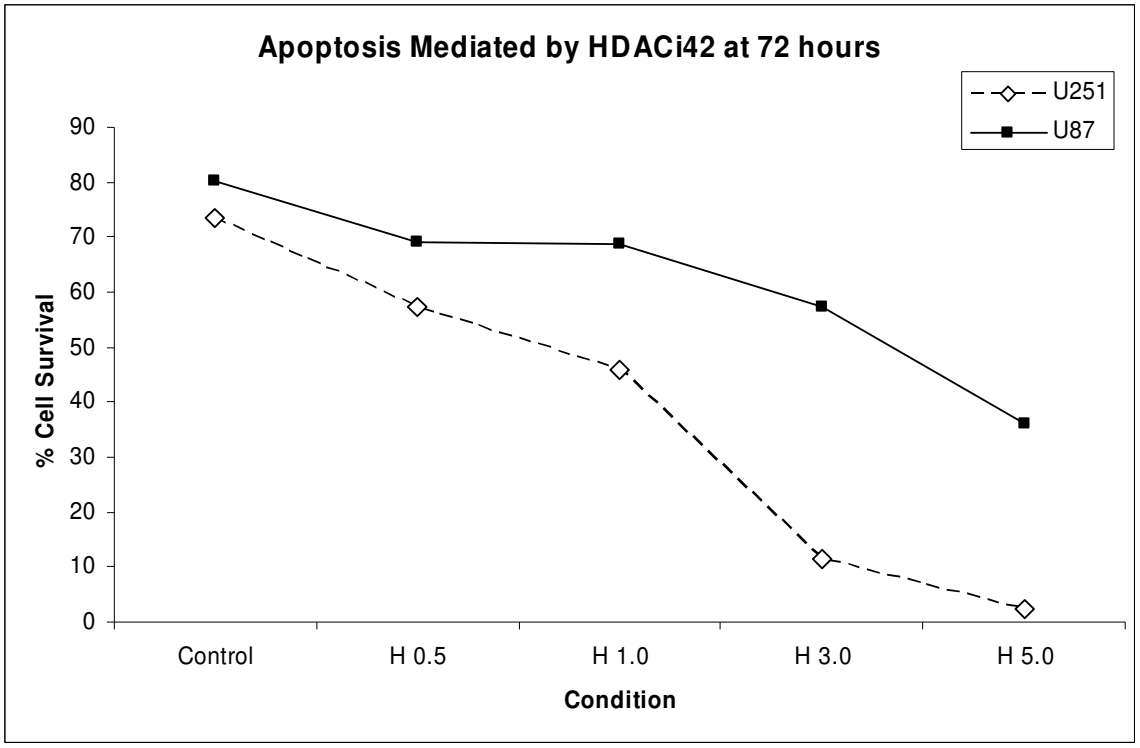

Figure 7. HDAC inhibitors induce apoptosis. Two GBM cell lines, U87 and U251, were grown in the presence of varying concentrations of an HDAC inhibitor, HDACi42. After 72 hours, apoptosis was assayed through flow cytometry, staining for Annexin V and TOPRO-3 (a DNA dye like propidium iodide). Double-negative cell events were interpreted as surviving cells. Apoptosis increased in a dose-dependent fashion in both cell lines.

Two cell lines, U87 and U251, were grown in increasing concentrations of HDACi42. At 72 hours, apoptosis was measured by flow cytometry, staining cells with a fluorochrome-conjugated antibody, Annexin V-FITC, and Topro-3, a dye that intercalates DNA strands. Annexin V binds phophatidylserine compounds that typically are found on the inside of cell membranes, but as the cell undergoes apoptosis, they accumulate on the outside. Thus, cells that exhibit Annexin V positivity are beginning apoptosis. Cells that have died by necrosis or apoptosis have permeabilized membranes, allowing Topro 3 to enter the cell and bind their DNA. Thus, cells that are positive for both Annexin V and Topro 3 are completely dead.

Data was analyzed, and the percentage of cells that were both Annexin V and Topro 3 negative--and therefore had not initiated apoptosis—were quantified.

Dose-dependent induction of apoptosis was observed in both cell lines. Increasing concentrations of HDACi42 induced increasing levels of apoptosis. Significantly, similar levels of apoptosis were not observed in each cell line. Different continuous cell lines were isolated from different patients. These different cell lines may possess different genetic and epigenetic irregularities, and these disparities may translate into different sensitivities to drugs. More mechanistic work to characterize the molecular differences between the cell lines is needed.

HDAC inhibitors reduce apoptotic threshold for standard chemotherapeutics

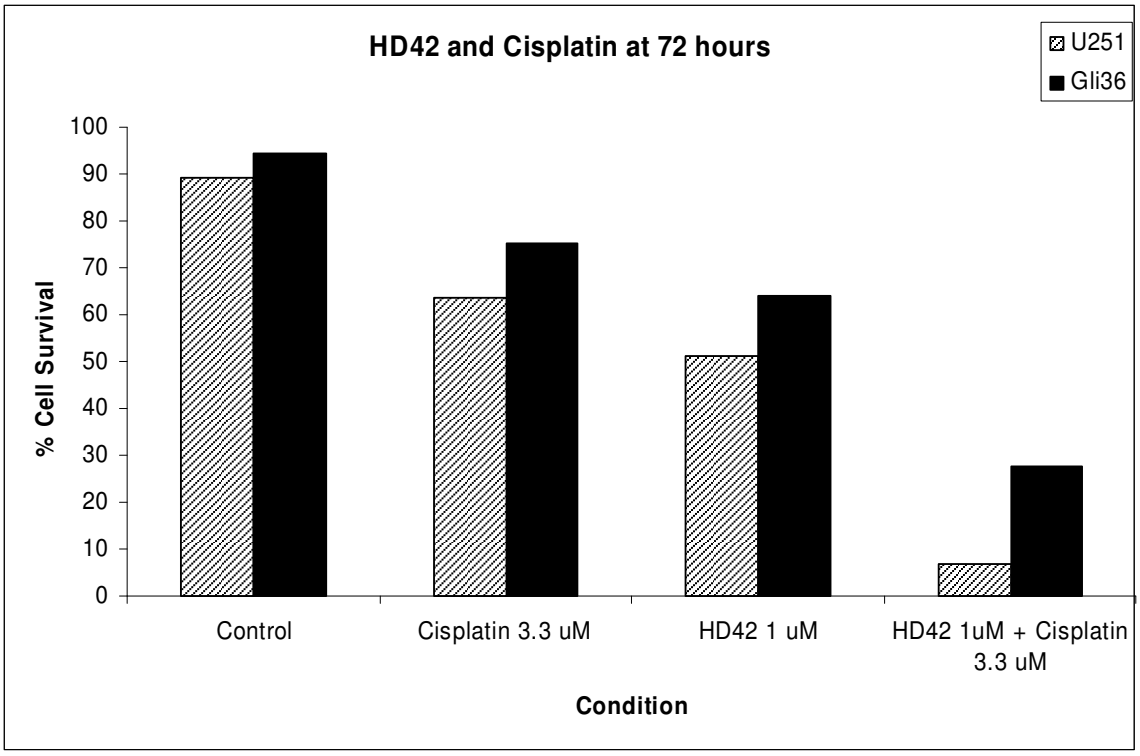

Figure 8. Reduction of apoptotic threshold by HDAC inhibitors for chemotherapeutics. Two GBM cell lines, Gli36 and U251, were grown in the presence of an HDAC inhibitor, HDACi42 at 1 uM, and the chemotherapeutic agent, cisplatin, at 3.3 uM. After 72 hours, apoptosis was assayed through flow cytometry, staining for Annexin V and TOPRO-3. Low concentrations of HDAC inhibitors sensitize GBM lines to standard chemotherapeutic agents.

Gli 36 and U251 MG cells were grown in sublethal concentrations of HDACi42 (1 uM) and cisplatin (3.3 uM), a common chemotherapeutic agent that damages DNA. Apoptosis was again measured using flow cytometry, and staining with Annexin V-FITC and Topro-3. In both cell lines, addition of a single compound induced apoptosis in a portion of the cell population. At 72 hours, cisplatin alone reduced cell survival from 90% to 65% in U251 MG cells, and from 93% to 75% in Gli 36 cells. HDACi42 alone reduced cell survival from 90% to 55% in U251 MG cells, and from 93% to 68% in Gli 36 cells. However, the addition of both compounds together led to a greater than additive effect, as cell survival in U251 MG cells was reduced to 5%, and Gli 36 survival was reduced to 25%.

These results indicate that HDACi42 can sensitize cells to chemotherapeutics, due to several possibilities. First, the effect could be a non-specific, genome-wide result, as HDAC inhibitors could open up chromatin structures, making DNA strands more accessible to cisplatin-induced DNA lesions. Alternatively, the epigenetic effect of HDACi42 could derepress important tumor-suppressor genes. Such genes could affect numerous cell signaling pathways, rendering the cells more amenable to apoptosis. Importantly, these explanations are not mutually exclusive. However, to elucidate the mechanism of the second possibility, genome-wide screens would need to be performed to find upregulated genes, and these genes would need to be individually silenced to confirm their necessity to the process.

We concluded that HDAC enzymes play an important role in the regulation of apoptosis and cell cycle in glioblastoma cells. Because HDAC enzymes have been shown to associate with other enzymes with repressor activity (DNMT and PRMT5) to target and silence important gene regulatory gene products, we next sought to investigate the role of histone methylation via PRMT5 in GBM tumors.

PRMT5 over expression and tumor suppressor gene silencing in GBM lines. Total cell protein lysates were prepared from GBM and control cells with RIPA buffer. 30ug total protein was subjected to PAGE and western blots were performed with antisera specific for PRMT5 and a direct target gene of PRMT5, ST7. PRMT5 is ubiquitously over expressed among GBM cell lines and the tumor suppressor gene ST7 is silenced. Pal et al (2003) have shown that the tumor suppressor genes ST7 and NM23 are directly targeted and silenced by SWI/SNF complexes that contain PRMT5, HDAC and DNMT1. Chromatin immuno precipitation (ChIP) experiments have previously demonstrated ST7 to be directly repressed by PRMT5-associated SWI/SNF complexes (Pal et al, 2003). Resting B cells are negative control for PRMT5. Jijoye and LCLMS are two EBV+ transformed B cell lines that serve as the positive control for PRMT5.

Western blots need appropriate loading controls, to ensure an equal amount of protein is loaded in each well and that different levels of protein as indicated on the blot actually correspond to differing levels in the cells. Actin was used as this control; due to its role as a key member of the cytoskeleton, it is highly, universally expressed, and is affected minimally by treatment, so actin levels are generally proportional with protein levels overall.

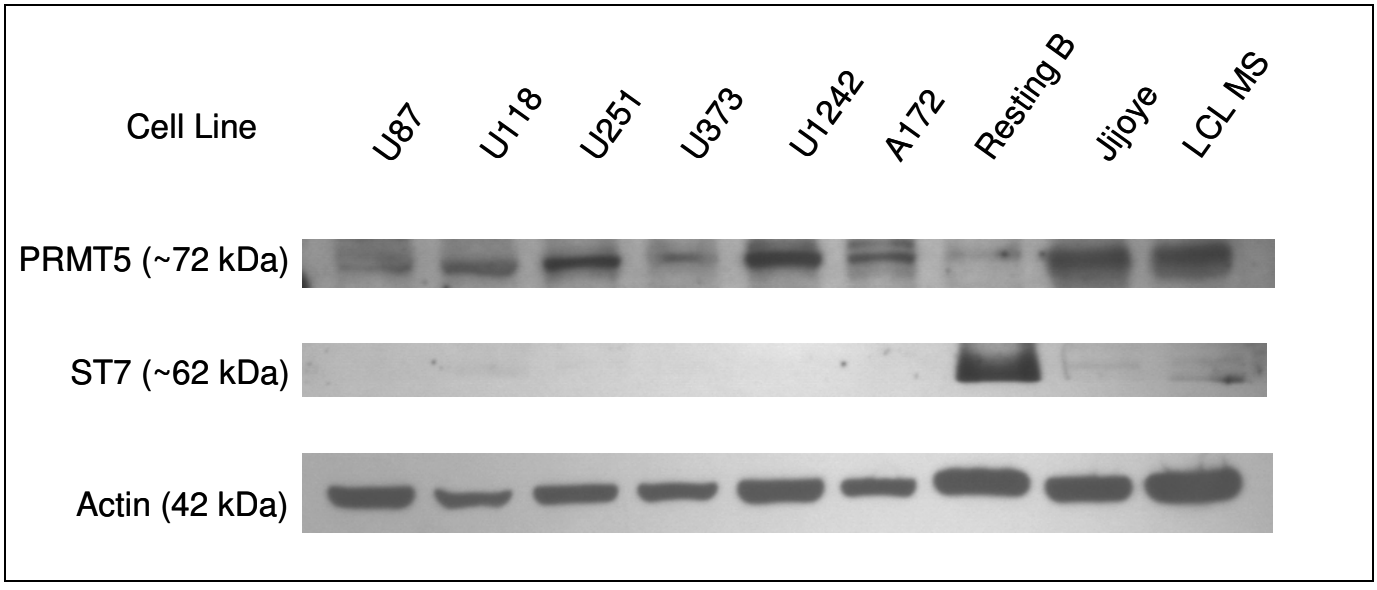

Figure 9. PRMT5 expression in a panel of glioblastoma multiforme lines. RIPA cell lysates of six GBM lines (U87, U118, U251, U373, U1242, and A172), as well as resting B cells, Jijoyre cells, and LCL MS cells, were made. An SDS-PAGE gel was run with 30 ug of protein in each well. Samples were probed for PRMT5, ST7, and actin (as a loading control). Actin indicated the samples were loaded equally, the resting B cells indicated the ST7 antibody worked correctly, and the Jijoye and LCL MS cell lines indicated the PRMT5 antibody worked correctly. PRMT5 was universally expressed in all GBM lines tested, and ST7 was universally silenced.

All six GBM lines (U87 MG, U118 MG, U251 MG, U373 MG, U1242 MG, and A172) expressed abundant levels of PRMT5, and importantly, within these lines, no expression of ST7, a tumor-supressor gene silenced by PRMT5, was observed. Resting B-cells, which were previously known to express minimal levels of PMRT5 and express abundant ST7, demonstrated that the anti-PRMT5 antibody did not bind aspecifically, and the ST7 antibody was functional. Jijoye and LCL 100, two B-cell tumor lines previously known to express PRMT5 and not ST7, demonstrated the PRMT5 antibody worked correctly. We concluded that PRMT5 is overexpressed in all GBM lines observed, and that this expression is correlated with ST7 silencing, although we have not yet demonstrated silencing of ST7 is caused by overexpression of PRMT5. We will be repeating ChIP experiments in GBM lines to prove that ST7 is directly targeted.

We next sought to silence PRMT5 using an siRNA oligonucleotide. Silencing PRMT5 with SiRNA. We have generated antisense and SiRNA reagents for evaluating the role PRMT5 plays alone and in concert with HDAC and DNMT enzymes with respect to tumor suppressor gene expression and cellular transformation.

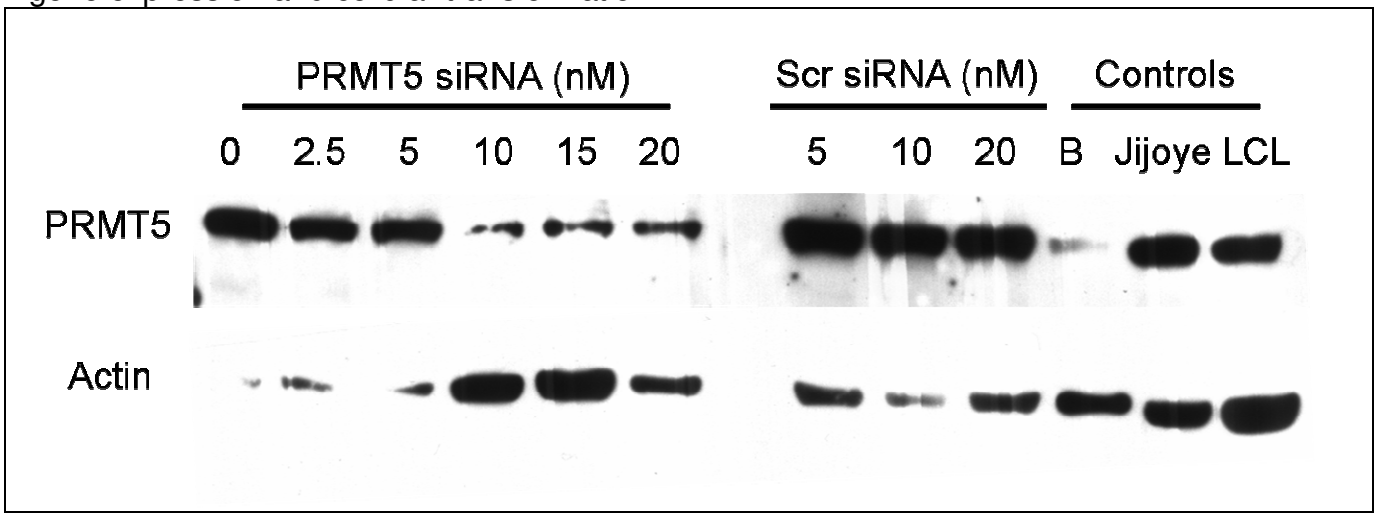

Figure 10. siRNA knockdown of PRMT5 in U251 MG cells. U251 MG cells were transfected with increasing concentrations of siRNA specific to the PRMT5 sequence, and increasing concentrations of a non-specific siRNA (Scr siRNA). After one week of growth, cells were lysed using RIPA buffer, and run in a SDS-PAGE gel, with 30 ug protein per well. Each sample was probed for PRMT5 and actin, as a loading control. Observed actin loading is inconsistent, possibly due to a transfer error, but loading was adequate at higher concentrations of pRMT5 siRNA, indicating PRMT5 knockdown occurred. Expression of PRMT5 in presence of non-specific siRNA indicated the sequence-specificity of the siRNA was necessary for knockdown. B cell, Jijoye, and LCL lysates indicated antibodies worked properly for Western blot.

U251 MG cells were treated with increasing concentrations of an siRNA antisense to PRMT5 mRNA. siRNA strands a small, double-stranded RNA sequences, which bind to Dicer, a riboendonuclease that cleaves the siRNA into single-strands. These single-stranded RNA's bind with another endonuclease, forming the RISC complex. The single-strand RNA antisense to the mRNA of interest binds to that mRNA, and the endonuclease in the RISC complex cleaves the mRNA. Consequently, genes can be silenced experimentally.

After the U251 MG cells were grown for one week after siRNA introduction, cells were lysed and run on a Western blot, probing for PRMT5 levels. Although the actin control bands at lower siRNA concentrations are not evident—probably due to a transfer error, loading bands at higher concentrations are evident. Consequently, we can conclude that the reduced expression of PRMT5 at 10 nM is due to silencing by siRNA.

Scramble siRNA was used as a control. Its sequence has no homology to any known gene, so it cannot, as far as is known, silence genes through a RISC complex. The abundance of PRMT5 in cells treated with scramble siRNA indicates that the mere presence of the siRNA chemically cannot knockdown PRMT5 levels; rather, a specific sequence antisense to PRMT5 is necessary. The three lanes on the right act as controls for antibody function in the Western blot and indicate that the PRMT5 antibody worked functionally and specifically.

Conclusions

We have concluded that HDAC does play an important role in the biology of glioblastoma multiforme. Moreover, mitigating its effects via chemical HDAC inhibitors such as HDACi42 and depsipeptide induces cell cycle arrest and apoptosis, while making cells more amenable to traditional chemotherapeutic treatments. We are currently working on clarifying the mechanism(s) that become operable during HDACi-induced apoptosis.

PRMT5 has been shown to be expressed in all GBM lines investigated, and correlates with ST7 silencing. Moreover, PRMT5 can be silenced using specific siRNA compounds. More recent work in our lab has demonstrated that PRMT5 cooperates with HDAC enzymes to silence at least 5 tumor suppressor genes including 3 in the retinoblastoma family (Rb, p105, p107 and p110). This will have great implications if we determine that ST7 and Rb family proteins are repressed by PRMT5 assisted processes because development of inhibitors to PRMT5 may become active anti neoplastic agents. We are currently exploring a wide screening of multiple natural products and small molecules to discover candidate compounds with PRMT5-inhibitory activity.

Our continued work will focus on several specific aspects of the epigenetics of GBM. First, we will investigate the mechanism of HDAC and its inhibition in the cells, testing for the overall levels of difference HDAC enzymes, such as HDAC2, and for their association with Swi/SNF complexes in GBM. Second, we will use Western blots to measure specific histone acetylation and nonspecific protein acetylation under HDACi treatment, and find which cellular phenotypic changes correlate with specific histone acetylation.

Third, we will investigate the various phenotypic changes associated with inhibition of PRMT5, and whether its silencing has any effect on proliferation, apoptosis. Moreover, we will investigate whether PRMT5 acts on ST7 directly, as in B-cell tumors, through chromatin mmunoprecipitation assays.

Fourth, we will investigate whether other epigenetic processes such as DNA methylation are important in GBM and if enzymes with DNA methylation activity are associating with PRMT5 and HDAC-containing SWI/SNF complexes.

Finally, we will investigate whether combination therapy targeting multiple epigenetic processes can induce a synergistic response in GBM cells, serving as an effective treatment using minimal doses of all agents.

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