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A MAJOR DOUBLE STRAND REPAIR PATHWAY AND CANCER-ASSOCIATED CIRUCULATING PROTEINS ARE EFFECTERS OF EPIGENETIC REVISION

by

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B.S. University of Florida, 2012

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ABSTRACT

DNA methylation is a vital epigenetic process that acts as a major control mechanism for gene expression. In addition to its essential role in many normal cellular processes, it is also implicated in a wide variety of disease states and processes including cancer. Along with genetic mutations, aberrant DNA methylation patterns, specifically the inappropriate DNA methylation or demethylation of CpG residues, may activate oncogenes or suppress tumor suppressor genes, respectively. These changes can generate or facilitate the progression of tumorigenesis and tend to accumulate throughout the development of cancer. Although they play such a major role in cancer and in other diseases, it remains unclear what causes these epigenetic revisions to occur. This dissertation will focus on uncovering mechanisms that are sources of epigenetic revision, specifically as they relate to cancer.

Due to rapid cell division and increased DNA damage, cells are increasingly dependent on DNA repair as they continue on a path of tumorigenic progression. We hypothesize that DNA repair, specifically the repair of DNA double strand breaks (DSB) by Non-Homologous End Joining (NHEJ) may play a role in inappropriate epigenetic revision. Using a GFP reporter system inserted into the genome of HeLa cells, we are able to induce targeted DNA damage that enables the cells, after successfully undergoing NHEJ repair, to express WT GFP. These GFP+ cells were segregated into two expression classes, one with robust expression (Bright) and the other with reduced expression (Dim). Using a DNA hypomethylating drug (AzadC) we were able to demonstrate that the different GFP expression levels was due to differential methylation statuses of CpGs in regions on either side of the break site. Deep sequencing analysis of this area in sorted Bright and Dim populations revealed a collection of different epi-alleles that display patterns of DNA methylation

following repair by NHEJ. These patterns differ between Bright and Dim cells which are hypo- and hypermethylated, respectively, and between the post-repair populations and the original, uncut cells. These data suggest that NHEJ repair facilitates a rewrite of the methylation landscape in repaired genes, elucidating one potential source for the altered methylation patterns seen in cancer cells.

The Dim cells generated during this study are known to have a hypermethylated GFP gene that is correlated with reduced expression, allowing it to be used as a screening tool for hypomethylating agents. We used this tool to screen the blood serum of patients with head and neck squamous cell carcinoma (HNSCC). We found that the serum from HNSCC patients, but not from healthy individuals, contains some factor that causes hypomethylation in exposed cells. Further, we were able to identify this factor as a protein capable of effecting changes in DNA methylation, gene expression, and miRNA levels in the treated Dim cells. The novel concept presented in this study has immense implications on the study of cancer progression as it evidences circulating proteins, presumably released by cancer cells, which are able to effect gene expression in cells that are distal to the location of the cancer. Further, the fact that these proteins are in circulation makes them a potential target for use in diagnostics.

Changes in DNA methylation play a major role in the development of cancer and understanding the mechanisms by which this occurs could provide new therapeutic targets for preventing this process from contributing to tumorigenesis. This dissertation presents potential sources of epigenetic revision in cancer and thus provides answers to a major question that has yet to be answered in the area of cancer research.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE: INTRODUCTION

Epigenetics

An organism's characteristics are a result of two layers of information; the first is the message that is contained in their genetic code and the second is how that code is read and implemented. The study of the information contained in the genetic code is termed *genetics*, while the study of how the genetic information is expressed is termed *epigenetics*. Since all of an organism's cells contain the same genetic code (barring stochastic DNA mutations), it is epigenetics which ultimately shape the specific characteristics of a cell as they change and control gene expression levels.

This is accomplished by mechanisms that physically alter chromatic configuration to manage what genes are accessible to transcription machinery, including DNA methylation, histone modifications, or RNA-associated silencing. Genes that are available to transcription machinery are transcribed while those that are hidden are repressed, allowing cells with the same genetic makeup to have vastly different characteristics. Although epigenetic information is alterable, it is also heritable so that cells are able to produce daughter cells with the same gene expression profile. This process is essential for generating the different cell types with different functions that make up an organism.

DNA methylation

The first level of epigenetic regulation occurs by marking specific locations of the DNA strand itself using DNA methylation. DNA may be methylated on cytosine residues of CpG islands

by the catalytic activity of DNA methyltransferases (DNMTs). DNMTs catalyze the addition of a methyl group, which is donated by the cofactor S-adenosylmethionine (SAM), to the carbon at position 5 of the pyrimidine ring of cytosine residues to generate 5-methylcytosine (1). Methylation profiles are passed from parent to daughter cells by DNMT 1, a maintenance DNMT that copies methylation patterns of hemimethylated DNA from the template strand to the newly synthesized strand during DNA replication. DNMT3a and 3b act as de novo methyltransferases, adding methyl groups to previously unmethylated CpG residues. DNMT1 has also been shown to have some de novo methyltransferase activity under certain conditions (1,2).

DNA methylation may be classified as either invariant and stable (sex-specific imprinting) or metastable. In somatic cells, DNA methylation is metastable and changes with age (3), diet (4,5), environment (6), disease (7-10), or other external or intrinsic events (11). In these studies, we are examining somatic, metastable DNA methylation. This epigenetic modification is usually associated with gene silencing (12,13) as it interferes with transcription machinery and is recognized by proteins that recruit histone modifiers to condense chromatin, an action which blocks the accessibility of transcription machinery to the affected genes (14-16).

DNA methylation in cancer

Under normal conditions, epigenetic modifications serve to modulate gene expression during embryonic development (17) and genomic imprinting (18), differentiation (19), or in response to stimuli (20); however unscheduled changes in epigenetic modifications have been linked to numerous health conditions including neurodegenerative (9), cardiovascular (21), imprinting-related (22), and metabolic diseases (23) and are known to play a prominent role in the development of

cancer. Incorrect revision of the normal epigenetic landscape can cause inappropriate gene silencing of tumor suppressor genes or activation of oncogenes, a phenomenon that is seen in various types of cancer cells (8,12,24,25).

Cancerous cells arise in a multi-step process as a result of an upset in the balance of oncogenes and tumor suppressor genes that causes deregulation of normal epistasis. This imbalance may derive from genetic mutations that affect this balance; however, changes in expression levels can also be caused by inappropriate DNA methylation marks. Moreover, the addition or removal of post-translational modifications to histones can affect the access of transcriptional machinery to particular genes by opening or closing the chromatin, causing gene activation or repression, respectively (13,24,26,27). Acetylation of histones, catalyzed by histone acetyltransferases (HATs) causes activation of genes by reducing the affinity of histones for DNA and by acting as a docking surface for other histone remodeling proteins (28). Histone deacetylases (HDACs), including Sirtuins, work in reverse to repress transcription along with histone methyltransferases (29).

Both hypermethylation and hypomethylation can contribute to tumorigenesis when they occur at inappropriate positions in the genome. DNA hypermethylation is generally associated with gene silencing while hypomethylated is associated with gene activation (8,24). Changes in the cellular DNA methylome resulting in hypermethylation of tumor suppressor genes including APC, BRCA1, E-cadherins, DAPK1, hMLH1, p15, Rb, MGMT, and p16INK4a have been documented in tumors from a variety of cancers including breast, colon, gastric, ovarian, lung, brain, ovarian, renal, kidney, prostate, thyroid, lymphoma, and leukemia (8,13,24). Hypomethylation has also been shown to contribute to tumorigenesis by over-activating oncogenes and contributing to genetic instability and structural changes by promoting an open chromatin state (8,30)

Coordination of genetic and epigenetic processes

Cancer cells coordinate genetic mutations and epigenetic revision in order to promote carcinogenesis. According to the two-hit hypothesis, a tumor suppressor gene must be deactivated in both alleles in order to cause cancer. HCT116 colon cancer cells were found to have genetic mutations in one allele of both CDKN2a and MLH1 and epigenetic silencing of the second allele, demonstrating the collaboration of genetic mutation and hypermethylation in achieving the second 'hit' to cause loss of heterozygosity and inactivation a tumor suppressor gene (8). Both hypermethylation and hypomethylation can also contribute to cancer progression by promoting mutagenic processes. This is observed in the silencing of genes involved in DNA repair via hypermethylation, which results in defective repair pathways and increased DNA mutagenesis (26,31). In addition, hypomethylation can cause over expression of an oncogene due to the loss of genomic imprinting that results in the expression of both alleles instead of only one allele (32,33), and hypomethylation of LINE retrotransposons facilitates insertion mutagenesis (34,35). Further, mutations or dysregulation of epigenetic regulation proteins can propagate DNA methylation changes in other areas of the genome.

Although DNA methylation changes are known to play a major role in the development and progression of cancer, the mechanism(s) involved in effecting these changes remain unresolved. It is the goal of this dissertation to investigate and identify sources that may generate and propagate epigenetic revision during the process of carcinogenesis.

CHAPTER TWO:

Introduction

DNA damage and repair

In order to pass the G2 checkpoint and enter mitosis, cells must repair any DNA damages that have occurred. Double stranded breaks in DNA (DSBs), in which breaks occur in both strands of the DNA double helix in close proximity to one another, are the most dangerous damage for a cell (36,37). These breaks can be caused by both exogenous and endogenous sources including reactive oxygen species, ionizing radiation (38), replication fork collapse (36,39), or the faulty action of nuclear enzymes such as topoisomerase II (40,41).

Regardless of origin, DSBs are fatal to the cell if not repaired. When faced with such a damage, the cell must repair the damage in order to survive or continue dividing (36). Double strand breaks are repaired by one of two pathways in Eukaryotic cells (42). During S and G2 phases of the cell cycle, when DNA has been replicated and exists in pairs of sister chromatids, the cell is able to fix the breaks with the high fidelity process of Homology Dependent Repair (HDR) (43,44). This process involves resection of one strand of a broken end to produce a single stranded overhang that can invade the helix of the sister chromatid. Polymerase then uses the sister chromatid to fill in sequence on the broken ends and the strands are resolved to separate, complete sister chromatids (40,45).

During the rest of the cell cycle, or in non-dividing cells, no identical sequence template is available to allow HDR to proceed, so the cell turns to the faster but more error prone process of

Non-Homologous End Joining (NHEJ) (46,47). This process proceeds through recognition and binding of the broken ends by Ku 70-80 proteins and DNA-PKcs (48). Together, this complex has a role similar to that of Proliferating Cell Nuclear Antigen (PCNA) during replication as it acts as a docking platform for other proteins. During repair, these other proteins are nuclease, polymerase, and ligase complexes needed to process the repair. The DNA-PKcs complex with Artemis has 3' and 5' endonuclease as well as 5' exonuclease activity, allowing it to process a diverse array of damaged DNA ends. Polymerases μ and λ are also able to interact with the complex, allowing flexible and template-independent synthesis. The processing of DNA ends during NHEJ is not fully understood and is not the same for each break; even identical breaks in the same location show variation in end processing (48). Blunted DNA ends are subsequently ligated through the action of XLF:XRCC4:DNA ligase IV complex. Although the immediate threat to the cell is averted by repair of the DSB, repair by NHEJ often results in deletions or frame shifts in the repaired area as a result of end processing. This process is a major source of DNA mutation in arrested cells (49). Despite its limitations, the quick kinetics and ability to repair without a template make NHEJ the repair pathway of choice in cells outside of S and G2 and in non-dividing cells. It is the predominant DSB repair pathway in animal cells since it occurs throughout the cell cycle (46,47).

Methylation, repair, and HDR

Studies of HDR have determined that, following repair, some cells exhibit robust expression of the repaired gene while others show low expression levels. It was determined that these expression classes arise as a result of epigenetic reprogramming, more specifically, altered CpG methylation at the repair site (50-52). In addition, specific DNA methyltransferases have been found

to localize at DNA repair sites (50). During S phase, DNMT1 methylates hemimethylated DNA during replication, copying the methylation profile of the parental DNA strand to the daughter strand, a process which is also ongoing when DNA methylation is altered post-HDR (50,53). Thus, DNA methylation exists in a triad of dynamic, interworking processes along with DNA replication and HDR.

The mechanism for DNMT-mediated methylation at repair sites following NHEJ, which occurs independently of DNA replication, is less well understood despite being a prominent DNA repair pathway in animal cells. We address this topic in the current paper. Specifically, in this work, we report the following observations. First, NHEJ repair pathway attended by DNA methylation revision in somatic human cells. Second, specific methylation sites map on the repaired gene to sites that are distinctly different from those seen in the HDR pathway (50). Third, we show that epigenetic revisions driven by NHEJ are stably inherited. In this work, we used a neutral gene to report alterations in DNA methylation to ensure that selective pressure post repair would not influence our ability to track the NHEJ descendants. Collectively, the data supports the notion that the prominent DNA repair pathway in animal cells is a source of genetic diversity but also a source of epigenetic (or gene expression) change in cases where a wild type allele is recovered post-NHEJ.

Materials and Methods

Cell culture

The stable HeLa cell line was cultured in RPMI medium with L-glutamine and supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were grown in at 37°C at 5% CO_2.

Stable cell line

HeLa cells were transfected with a Tet-On inducible gene expression system (Clontech) and set to target the I-Sce1 gene according to the Tet-On system manual. After generation of a stable Tet-On cell line, the cells were transfected with the GFP reporter construct for NHEJ provided by the Gorbunova lab (54). These cells were grown under selective pressure with Geneticin (G418) to generate the stable iHN20.22 cell line containing the reporter construct and a tetracycline inducible I-Sce1 gene.

Tet On promoter activation

IHN20.22 cells were treated with 1μg/mL doxycycline for varying durations to determine the optimal induction time. It was determined that a 24-hour pulse of doxycycline was sufficient to induce the NHEJ system, so this was used in future experiments.

FACS analysis

IHN20.22 cells were trypsinized and centrifuged at 1000xg for 5 minutes and then and re-suspended in PBS at a density of 10^6 cells/mL. Live cells were selected using a plot of SSC-A vs. FSC-A, and GFP positive cells were identified using a plot of FSC-A vs FL1A-A. The FL1-A histogram was then used to identify and gate the distinct populations of low and high expressing cells.

Live-Cell Imaging

Cells were seeded at low density in glass bottom culture dishes (MatTek) induced with Dox for 24 hours and then placed into an incubation chamber that is part of a Perkin Elmer UltraVIEW VoX 3D Live Cell Imaging System attached to a Zeiss Axio Observer Z1 inverted fluorescence microscope. Images were taken every 5 minutes for the next 48 hours and then analyzed using Volocity Imaging and Analysis software (Perkin Elmer).

Drug treatments

IHN20.22 cells were plated at low confluence and induced with doxycycline for 24 hours. After 24 hours, media was removed and replaced with fresh media and cells were given a daily dose of 1μM 5-AzadC or 1% DMSO control for 48 hours (unless otherwise noted) and then were harvested for FACS analysis.

Cell sorting

Dox induced IHN 20.22 cells were treated with dox for 24 hours and then grown under normal culture conditions until the GFP expression levels stabilized. Cells were then harvested and re-suspended in median containing RPMI, 1% penicillin-streptomycin, 20mM Hepes buffer, and 2% FBS at a density of $3x10^6$ cells/mL. Cells were run through FACSAria flow cytometer and sorted by using the FSC-A and FITC-A plot to gate GFP positive cells and then using the histogram of FITC-A to select 'dim' and 'bright' populations. Two sorted populations (Bright and Dim) were collected in a tube containing media supplemented with 30% FBS and then transferred to culture dishes containing culture medium (RPMI supplemented with 10% FBS and 1% Penicillin-streptomycin) at 37°C at 5% CO_2.

Bisulfite Converted DNA preparation

Genomic DNA was extracted from iHN20.22 cells (uncut) as well as the sorted bright and dim populations using Wizard genomic DNA purification kit" (Promega). 100ng of extracted DNA was used for bisulfite conversion using the Epijet Bisulfite Conversion Kit (Thermoscientific). Regions on either side of the repair site in bisulfite converted DNA were amplified using Phusion U Hot Start Polymerase (Thermoscientific) and 5 sets of primers (Table 2). The 5 PCR fragments of each sample (uncut, sorted Dim, and sorted Bright) were purified using GENEjet PCR purification kit (Thermoscientific).

Bisulfite Sequencing

These samples were transferred to the core sequencing facility at Sanford Burnham at Lake Nona, Orlando Florida. Illumina's Truseq ChIP Library Preparation kit was used to prepare a total of 15 libraries (5 fragments each for 3 samples: uncut, sorted Dim, and sorted Bright) from 10ng of input DNA. Quality and quantity of the libraries were analyzed using an Agilent Bioanalyzer and Kapa Biosystems qPCR. The Multiplexed libraries were pooled and subjected to Paired-end 2x250-bp sequencing using one flow-cell of a Miseq sequencing instrument.

Methylation analysis

FastQ files were subjected to quality check using FASTQC software (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc). Then, paired-end reads from the sequencer platform were merged together using PEAR tool (55) with a minimum of 40 overlapping residues as threshold (mean PHREAD score of at least 33) and merged FastQ files were converted in Fasta using Prinseq (56). To analyze the methylation status of each amplicon, we used AMPLIMETHPROFILER (57) specifically designed for deep targeted bisulfite amplicon sequencing of multiple genomic regions. This pipeline is freely available at https://sourceforge.net/projects/amplimethprofiler and is organized as follows: first, it recognizes corresponding target region discarding PCR artifacts and reads that do not match expected lengths; then, reads are aligned to the corresponding bisulfite-converted reference using BLASTn (58). We used very stringent parameters: fragment length threshold, 50%; threshold alignment primers, 80%; bisulfite conversion efficiency, 99% and threshold alignment to reference, 50%. The pipeline output format reports the methylation status for each CpG dinucleotide coded 0 as non-methylated, 1 as

methylated, and 2 if the methylation state cannot be assessed. We use this output to perform the analysis. Quantitative methylation average for each site is represented by the ratio between the number of non-converted bases at that site and the total number of mapped reads. The abundance of each of the 2NCpG distinct epialleles (where NCpG stands for the number of CpG sites in the analyzed region) was evaluated for each sample by counting the number of passing filter reads containing that epiallele. Qualitative methylation analysis was performed using Qiime (59), which includes: 1. a "summary", the number of profiles present in each input sample; 2. a "taxa_summary_plots" , the information on the distribution of methylation profile classes; 3. "alpha diversity" , the five alpha diversity metrics for each sample: a. number of different methylation profiles in the sample; b. shannon entropy; c. simpson index; d. Chao 1 index ; e. number of singletons. Such metrics were computed through a rarefaction procedure to take into account biases derived from variable sequencing depth and; 4. "beta Diversity", the distance between samples in terms of composition of their methylation profiles, measured by Bray–Curtis dissimilarity: 5. PCoA Principal Coordinates analysis.

Results

A neutral reporter system for analysis of epigenetic revision during NHEJ.

In order to study the NHEJ repair pathway, we have generated a HeLa cell line containing a GFP based reporter construct (54,60) (Figure 1). The construct contains a CMV driven GFP gene that was interrupted by a rodent Pem1 intron. Within this intron, an adenoviral exon was added that is flanked by two restriction sites for the megaendonuclease, I-Sce1. There are no I-Sce1 sites in the

human genome; Thus DSB are target to these twin sites. The presence of the viral exon disrupts the gene, and as a consequence, the cells are GFP negative; however, when I-SceI is introduced to the cells, the adenoviral exon is excised by two DSBs and repaired by NHEJ. Since removal of the exon allows the construct to generate WT GFP after splicing, cells have effectively undergone NHEJ repair and some sub-fraction of the cells will be GFP positive (since NHEJ is error prone, we cannot score mutant alleles with this assay based on GFP expression). In addition, there is no homologous DNA template for the repair event; therefore, this is a dedicated NHEJ repair process and HDR cannot proceed under these conditions. To improve accuracy and penetrance of the system, the gene for I-SceI gene has been placed in these HeLa cells under control of a Tet-On promoter, thus, the system is doxycycline inducible.

A segregation of expression classes following repair by NHEJ.

The NHEJ reporter system was tested by adding Doxycycline (Dox) to the media of the IHN20.22 HeLa cells. We note that other clones tested behaved similarly; however, some clones displayed higher backgrounds, probably due to leaky I-Sce1 expression in the absence of Dox. IHN20.22 was selected since this clone exhibited low levels of GFP positive cells in the absence of Dox. I-Sce1 induction was quite robust in IHN20.22 cells and within a few hours after Dox addition, Western blots showed the presence of prominent amounts of I-Sce1 protein (data not shown). At 24 hours post-Dox, GFP expressing cells could readily be seen by live imaging or fluorescent microscopy. GFP levels could also be measured using flow cytometry (Figure 2). The percentage of GFP positive cells steadily increases over time with continuous exposure to Doxycycline, while in negative controls (no Dox) the percentage of GFP positive cells stays well

under 1% which we attribute to leakiness of the Tet-On promoter, as noted. After a 24-hour pulse with Dox, the percentage increases and then peaks at about 5-7% after four days (Figure 3). The emergence of GFP positive cells can be observed via live imaging during the 72 hours following induction with Doxycycline (Figure 4) as a GFP negative cell (circle, top image) undergoes NHEJ repair and begins to express WT GFP as time progresses to the second image. Since the post-repair cell now contains a WT GFP gene, division produces two GFP positive daughter cells in the last image. By limiting the expression of I-Sce1 to 24 hours, the pulse-chase experimental schematic allows for focused investigation of the processes that occur following a DSB without the added variables of continuous DSB induction and continuous Dox treatment. Note that the scatter plots in Figure 3 appear to contain dual populations of GFP positive cells (dashed rings). The two populations, which appear to differ in total GFP expression levels were more clearly observable after Dox induction with I-Sce1; however, in the negative controls, this heterogeneity was observed. This was examined in more detail in order to understand the underlying basis for this observation.

GFP Expression is heterogeneous in repaired cell populations following NHEJ.

The histogram of GFP positive cells reveals the emergence of two expression classes with differing GFP intensities. One class expresses GFP robustly while the other maintains lower expression levels. We refer to these populations as Bright and Dim, respectively (Figure 5). To determine if the differing expression classes may be a result of DNA methylation, we tested whether the expression classes were altered by the DNA hypomethylating drug, 5'-Aza-2'-Deoxycytidine (AzadC). This drug acts by inducing a stable, covalent complex between the methyl's and DNA (50,61-63). The result is hypomethylation of the genome due to the sequestering of DNMTs that are

covalently bound to DNA. This prevents further methyltransferase action and effectively inhibits the overall DNA methylation of the cell. Based on this known mechanism, multiple cell divisions are required in order to observe genome-wide hypomethylation, which is manifested in the daughter cell population.

When IHN20.22 cells are induced with Dox and then treated with AzadC, there is an obvious shift of cells from the low expressing Dim population to the high expressing Bright population (Figure 6). The conversion of 'dim' cells to 'bright' cells by the hypomethylating drug suggests that the repaired GFP gene in the low expressing pool is a direct result of DNA methylation either during or soon after NHEJ repair. In the absence of AzadC, the Bright population of cells initially decreases over 4 days then appears to stabilize (Figure 7, -AzadC). In contrast, cells treated with AzadC displayed a clearly different trend (Figure 7, +AzadC). The drug reverses the loss in Bright cells, probably due to the ongoing conversion of Dim cells into the Bright pool. In either case, a few days after the damage/repair event, the proportions of cells in Bright and Dim cell populations remain fairly stable as cells are passaged (Figure 7); however, addition of AzadC to cells that are 24 days post-repair results in a sharp increase in the percentage of high expressing cells (Figure 8), supporting the notion that the expression difference is likely due to post-repair methylation as opposed to an off target drug interaction during the repair process. Further, the extent to which AzadC causes the shift from low to high GFP expression occurs is dose dependent (Figure 9).

Since it appears that the expression classes eventually become stable, presumably due to stable methylation marks (which are heritable) we next attempted to isolate pure populations of Dim and Bright cells to make the analysis more tractable. Before treatment, sorted Dim cells appear as a relatively homogenous population in a uni-modal distribution (Figure 10A) with relatively low levels

of GFP expression (labeled as the "P1" pool). Note that while the distribution is broad, it is nonetheless uniform with very low levels of bright GFP expressing cells (P4). Following AzadC treatment of the Dim cell pool, a new population emerges in the high expression range (Figure 10A, right panel "P4" pool). As with sorted Dim cells, sorted Bright cells also show a single peak of GFP expression (Figure 10B); however, these cells are far more homogeneous (compare Figure 10A, B). Since the Bright cell pool also shifts perceptibly to the right in the presence of AzadC (Figure 10B, right panel, P4), we conclude that the bright pool contains cells with some degree of DNA methylation (which is removed by AzadC) (Figure 3B). This experiment clearly evidenced the conversion of Dim cells to Bright cells by a DNA hypomethylating drug. Live imaging reveals that the information regulating the GFP expression level of post-repair cells is passed from parent to daughter cells as the cells divide (Figure 11). The cause of the silencing is heritable but reversible, pointing once more to DNA methylation as the source of the gene expression variation.

The availability of relatively pure populations of Dim and Bright cells makes it possible to use bisulfite DNA sequencing to interrogate methylation sites before and after NHEJ. Thus, in addition to validating the presence of epi-alleles in Dims and Brights, bisulfite DNA sequencing of the post-repair populations makes it possible to map these epi-alleles relative to the I-Sce1 cleavage and repair site. Bisulfite sequencing confirmed that the GFP gene is hypomethylated in sorted Bright cells and hypermethylated in sorted Dim cells. Specifically, changes occur in regions both up and downstream of the break site (Figure 12). Interestingly, the methylation status of CpGs in the region directly flanking the break site is not affected by the process. This suggests a coordination of methylation and repair proteins to affect methylation patterns in specific areas around the site of DNA damage.

Different DNA methylation patterns mark Recombinant from Uncut cells.

The data so far demonstrate that NHEJ is spinning out new epi-alleles that are either over-written or completely revised from the parental (uncut) reporter DNA. Moreover, the expressability of the repair products correlates with the percent of DNA methylation in sorted Dim and Bright cells. The pyrosequencing analysis done thus far evaluates the average methylation levels for a single CpG site derived from physically different molecules; however, it does not consider the relationship between the different methylated cytosines present on the same molecule (epialleles). For this reason, we have analyzed the composition of methylated population (heterogeneity), by counting the number of different epialleles in the sample (haplotypes) obtained from deep sequencing analysis of the amplicons.

The DSB region was divided into 5 segments (3 upstream and 2 downstream regions from the DSB site, (Figure 13). Deep quantitative (Taxa, PCoA and Shannon Index) and qualitative (methylation profiles) analysis of amplicons with the same end was carried out. The results show that recombinant cells (both Dims and Brights) and uncut parental cells have the same types of methylated species (un-methylated, mono, bi and tri-methylated) but with different compositions (Figure 13). In fact, the Bright cells appear to be rich in unmethylated species compared to Dims and Uncut in each region, confirming that high levels of GFP expression in this population are largely unmethylated (marked by an asterisk in Fig. 5A). Moreover, Principal Coordinates Analysis reveals that Dim and Uncut parental DNAs have a smaller euclidean distance compared to Bright (variation 96% *vs* 4%), highlighting a common origin for Recombinant (i.e., NHEJ repaired) cells, followed by a de-methylation event of the Bright cells (Figure 14). We also analyzed the diversity index (Shannon Index) in order to evaluate the evenness of the species. Except for the region 1

(where Bright and Uncut are much more similar to the Dims), we observe that 2 upstream regions of DSB site (Region 2 - 3) show a gradual increase in similarity between Dims and Uncut relative to the Bright cells (single asterisk*), while region 4 (downstream region of DSB site) shows a high similarity between the Recombinants (Dims plus Brights) compared to the Uncut (double asterisk **), which is lost in the region 5 (Figure 15).

Finally, we have performed a deep qualitative analysis to identify the differences between Recombinant and Uncut (parental) cells in terms of gain or loss of methylation (Figure 16). The differences between the percentage of Recombinant's epialleles compared to Uncut cells show that the Dim cells gain methylation marks in the region 1 (*di-methylated epialleles*), in the region 2 (*mono-, di- and tri-methylated epialleles*), and in the region 5 (*mono-, di- and tri-methylated epialleles*). In contrast the Bright cells lose methylated epialleles in these same regions. For example, in region 3, Bright cells appear to acquire new methylation (*di-methylated epialleles*) while DIMS lose methylation. Both Dims and Bright cells lose methylation (*tri-, di-methylated epialleles)* acquiring mono-methylated epialleles in the region 4. Knowing the different GFP expression between the two populations, we hypothesize that transcription may modify DNA methylation (or de-methylation) after DNA damage and NHEJ repair, remodeling the chromatin in units with different transcriptional efficiency (and in the process spinning out Dim and Bright cell populations).

Discussion

Epigenetic revision as a component of tumorigenesis

Despite their involvement in so many types of cancer, the root cause of DNA miscues is largely unknown. This study reveals a novel connection between two essential cellular processes that has a potential role in the development and progression of cancer. Deep sequencing analysis of post-repair DNA revealed both loss and of methylation in areas up and downstream of the break site (Figure 6B) that is correlated with up- or downregulation, respectively. The mechanism described here provides evidence for alterations in methylation profiles as a result of NHEJ repair, a process which could explain the epigenetic revision that is characteristic of cancer cells.

What is not clear is how universal and wide-spread NHEJ-mediated epigenetic revision paths are in a tissue context. In our model system, we used a gene that is not subject to selection; however, it stands to reason that silencing or activating a positive or negative growth-promoting gene could produce cells that have a growth advantage relative to surrounding normal cells (8,13,64). Growth promoting outcomes could clearly place the cell on a path toward a pre-cancerous condition. In contrast, silencing a pro-growth gene (oncogene) could result in the loss of a cell lineage. In either situation, tissue epistasis could be targeted with undesirable outcomes. What is intriguing is that even when an error-prone pathway such as NHEJ regenerates wild-type sequence, there is still a good chance for gene activation or silencing. Thus, mutating a tumor suppressor gene is functionally equivalent to epigenetic silencing the expression of the same gene, with the same dire consequences.

A new pathway for discovery of novel epi-therapeutic targets

Changes in DNA methylation have been shown to accumulate throughout the genome with the progression of cancer (64-67) and have been shown to positively correlate with tumor stage (66). Cancer cells have also been shown to acquire additional genetic mutations due to an increase in DSBs resulting from increase reactive oxygen species production, telomeric dysfunction, genomic instability, and replication errors (47,68). Although the increased DSB may give cancer cells a growth advantage by providing favorable mutations in key regulatory genes, it also makes them increasingly dependent on DSB repair in order to grow and proliferate at a high rate. If DNA methylation revisions occur during or soon after NHEJ, as demonstrated here, further DSB repair that occurs in rapidly dividing cancer cells may exacerbate the situation and could explain the accumulation of inappropriately methylated or demethylated genes that is characteristic of progressing cancer. The association of methylation revisions with tumor stage could enable their use as a cancer marker to predict prognosis of developing cancer and to predict responsiveness to specific therapeutics (33). Although methylation aberrations tend to accumulate with malignant progression, they have also been shown to be present in the early stages of pre-malignancy (8,67,69). This could make them candidates for use as a diagnostic tool for early detection of cancer cells (67,69). Further exposing the mechanism behind the overlap of these key processes could provide new targets for therapeutics to interfere with the progressive gain of epigenetic miscues by this mechanism and prevent tumor progression.

Intragenic DNA methylation and gene silencing

While methylation based gene silencing is typically associated with promotor CpG islands, studies have also shown that intragenic methylation may affect gene expression as well. Intragenic methylation in plant cells was shown to increase in more highly transcribed genes, presumably in an effort to prevent aberrant transcription at other nearby sites that can result when transcription machinery disrupts chromatin structure. Inhibition of DNA methylation in these cells caused up regulation of genes that were methylated only in the body of the gene and not in their promoters (70). Similar results were found in mammalian cells where intragenic methylation was shown to decrease gene expression and facilitate compaction of chromatin that is correlated with a reduced density of DNA polymerase II in the body of the gene (71). Further, another study concluded that a single methylated CpG in the intron of the PMP24 gene was sufficient to silence the gene. In addition to demonstrating the ability of intragenic methylation to silence genes, these studies suggest a connection between methylation and reduced elongation efficiency. This supports our findings that aberrant methylation in the body of the GFP gene causes silencing.

This also incites the idea that transcription may reshape the epigenetic landscape, a phenomenon that has already been demonstrated to occur following DNA repair by HDR (51). It was found that this process is mediated by Base Excision Repair (BER)-mediated demethylation (52). Principle Coordinates Analysis presented in Figure 5B revealed that Dim cells had a closer Euclidian distance to the original, uncut parental DNA than Bright cells. This data supports a model where the area surrounding the site of NHEJ is first methylated and then progressively demethylated over time with transcription, resulting in an array of epialleles with differing levels and locations of methylated CpGs. Future work will look further into this idea.

In summary, this study reveals a novel connection between two processes that have important roles in cancer development and progression, NHEJ and DNA methylation. This link provides a clue to one of the biggest unanswered question in this area of study: What causes the epigenetic revision that is pivotal to the multistep progression of cancer? While DNA damage and repair is stochastic, a damage event that results in epigenetic revision following repair and provides the cell with a growth advantage could direct the cell onto a path of tumorigenesis. This could explain why some genes exhibit inappropriate methylation patterns in some cancers but not others, as only genes that are beneficial to that particular tissue type would confer a selective advantage. Thus, methylation revision following DNA repair by NHEJ, the most predominant repair pathway in animal cells, fits the criterion to be the source of epigenetic abnormalities in cancer cells and could provide information for the development of new therapeutic strategies for preventing and stopping this process from contributing to tumorigenesis.

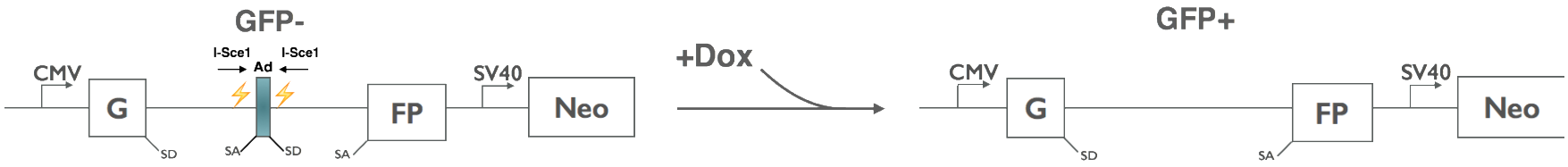


Figure 1. Reporter construct integrated into the genome of the IHN20.22 HeLa cell line.

The NHEJ reporter GFP gene contains a Pem1 intron interrupted by an adenoviral exon. Two I-Sce1 restriction sites allow the homing endonuclease to cut the DNA and excise the adenoviral exon to produce wild-type GFP following repair by NHEJ.

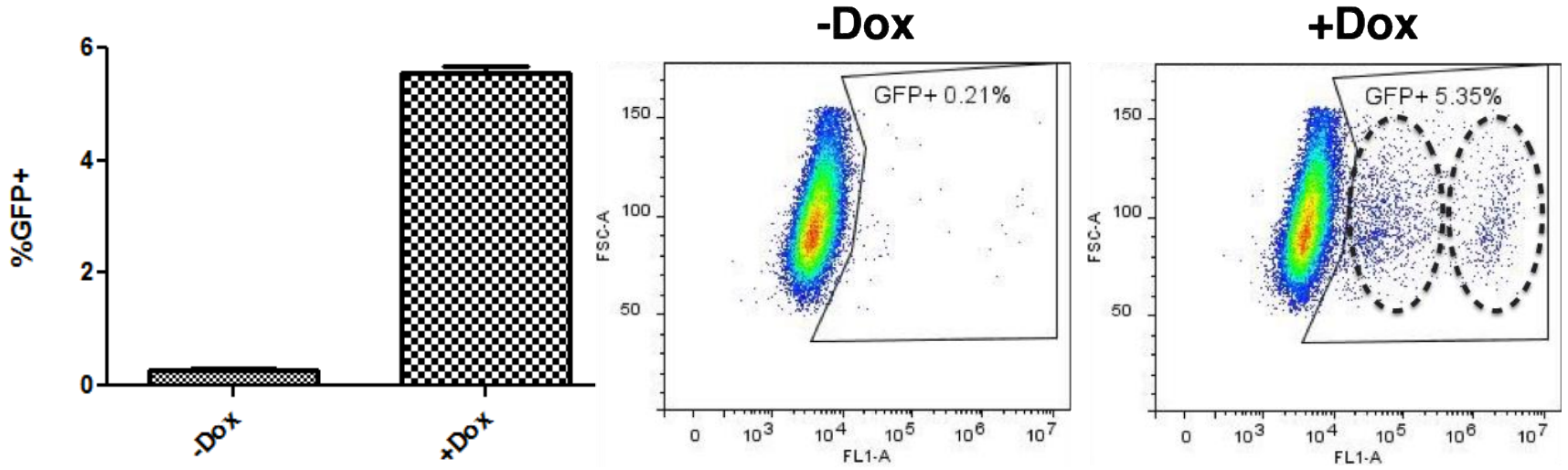


Figure 2. Generation of GFP positive cells following repair.

Cells were induced with dox for 24 hours and then the percentage of the population expressing GFP was analyzed using FACS. The circles on the "+Dox" plot indicate two separate GFP positive cell populations with differing expression levels of GFP

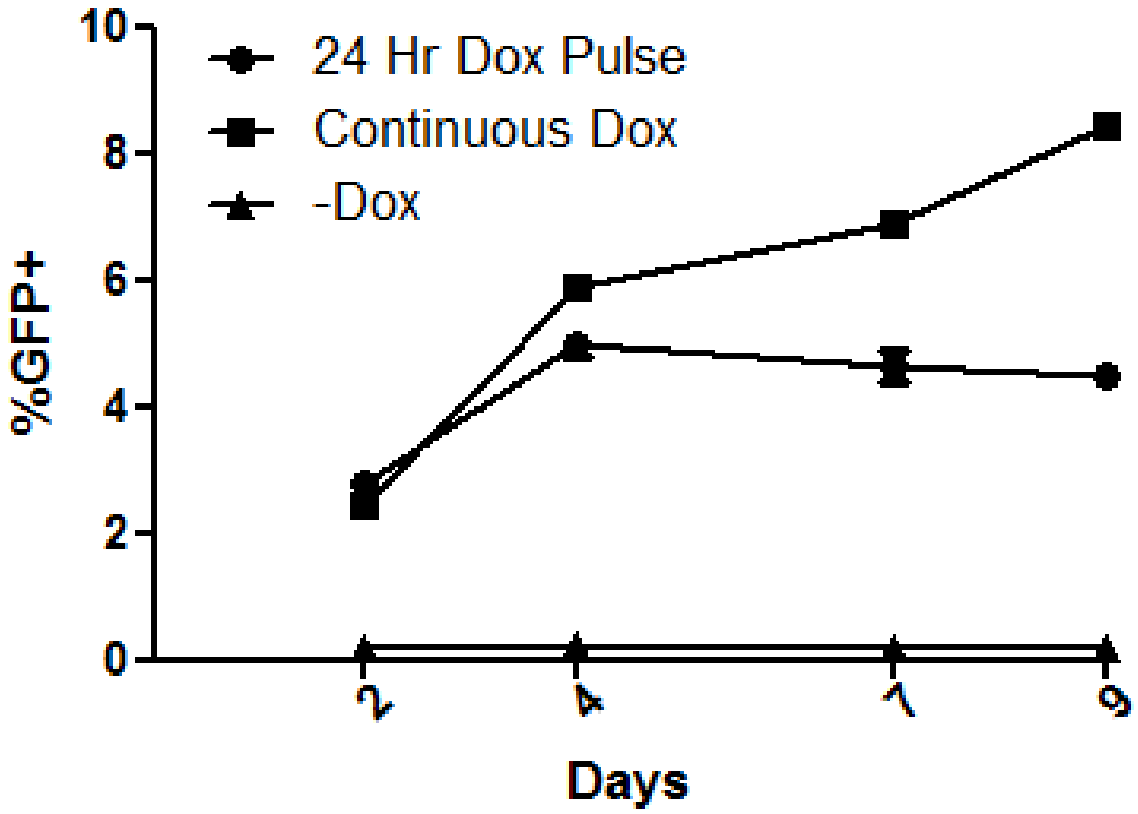


Figure 3. Time course analysis of GFP expression.

The percentage of GFP positive cells was analyzed by FACS over the course of 9 days, either following a 24-hour induction or with continuous exposure to doxycycline. The uncut cell line with no dox exposure was also analyzed to asses basal levels of GFP expression.

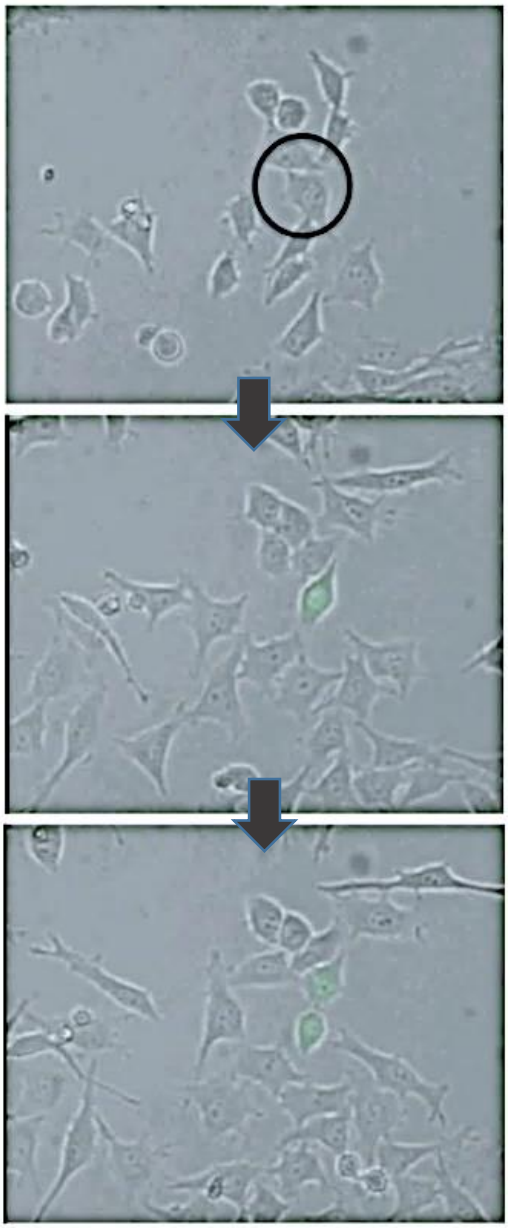


Figure 4. Live-cell imaging of GFP expression.

The onset of WT GFP expression in a single cell was observed during the 72 hours following a 24 hour Dox induction using live-cell imaging. The arrows indicate time progression and the black circle indicates the GFP negative cell that is GFP positive in subsequent images.

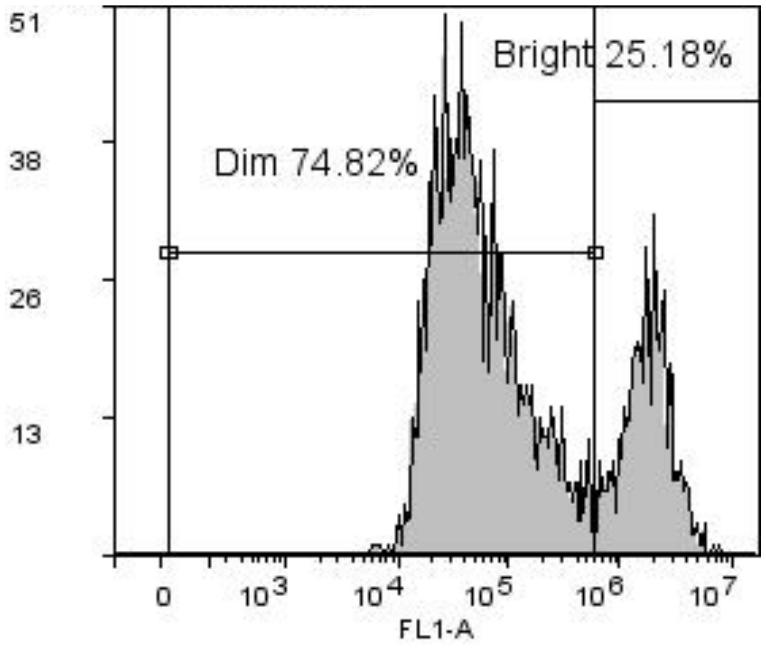


Figure 5. Histogram of GFP+ cells after induction with dox with gating for Bright and Dim populations.

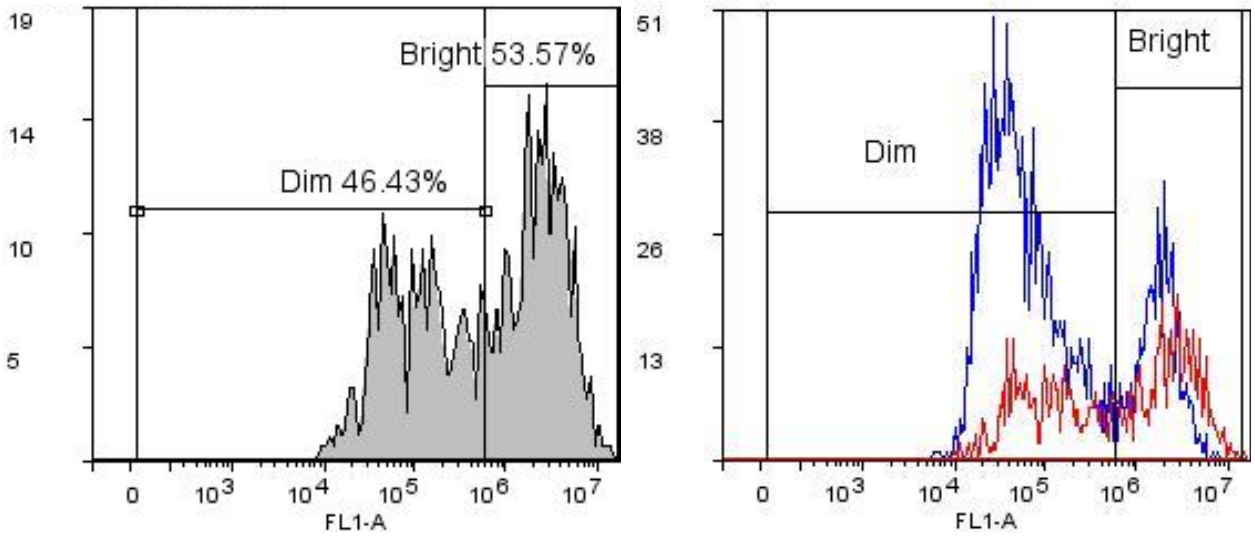


Figure 6. Hypomethylation by 5'Aza-2'-deoxycitidine.

IHN20.22 cells were induced with dox for 24 hours and then treated with a daily dose of 1μM AzadC for 48 hours. The percentage of cells with high (Bright) and low (Dim) GFP expression with and without AzadC treatment were quantified using FACS histograms of GFP positive cells. The second graph is an overlay of the histogram with (red) and without (blue) treatment with AzadC.

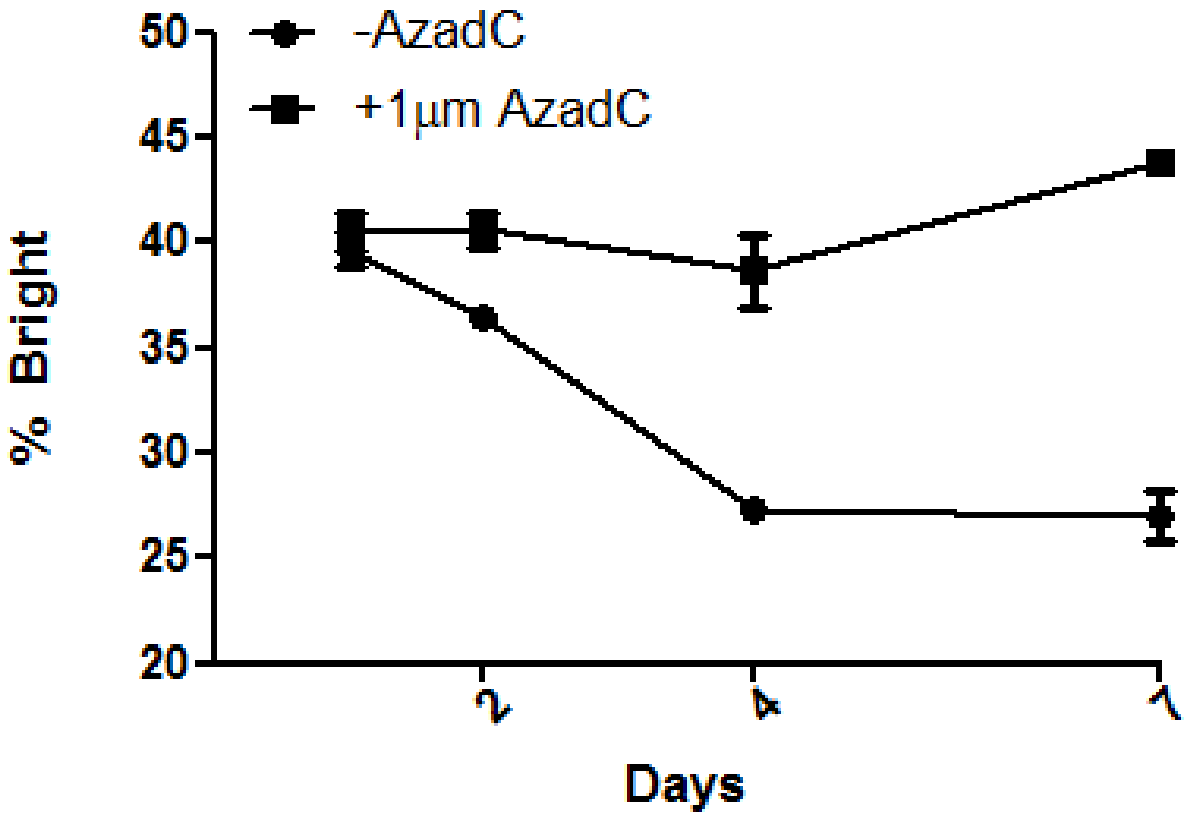


Figure 7. Characterization of the effect of hypomethylation by AzadC on the GFP expression level in post-repair cells.

GFP expression level over the course of 7 days is compared between cells with and without AzadC treatment. Cells were induced with dox for 24 hours and then given a daily dose of 1μM AzadC. The percentage of Bright cells was measured using FACS on days 1, 2, 4 and 7 following initiation of AzadC treatments.

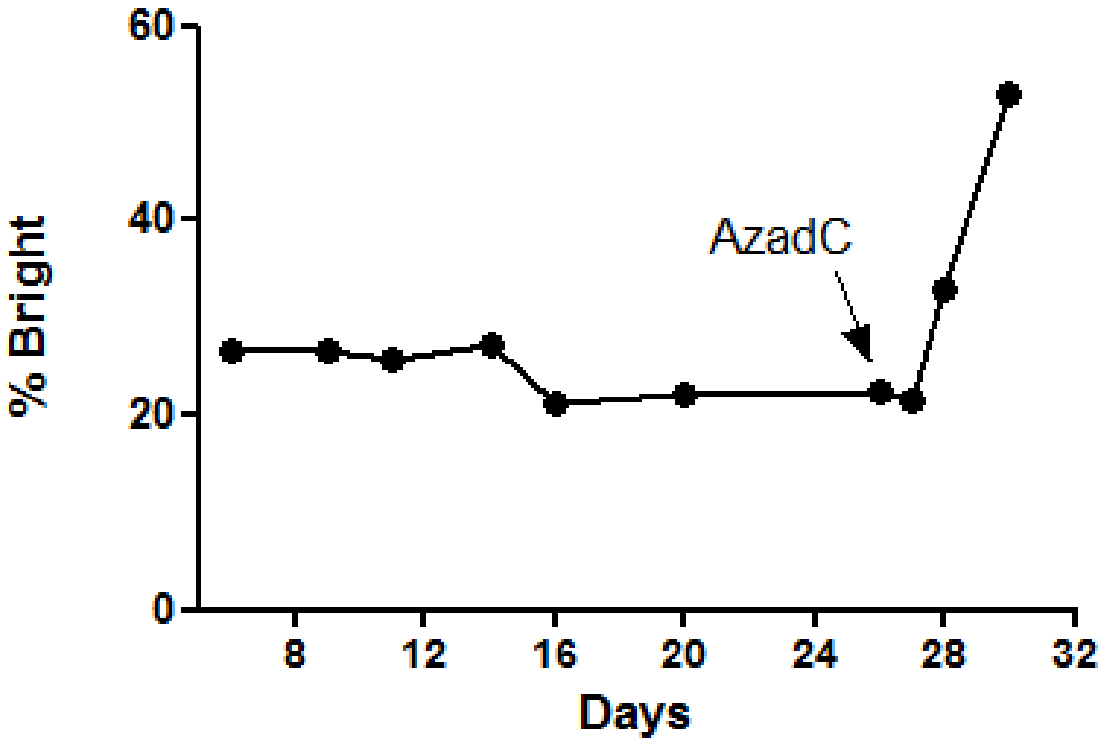


Figure 8. The effect of AzadC treatment on post-repair cells.

The distribution of cells in each expression class was observed in the days following NHEJ repair. As indicated by the arrow on the last graph, the cells were treated with 5μM AzadC on day 25 and then analyzed by FACS on day 26, 27, 28, and 30.

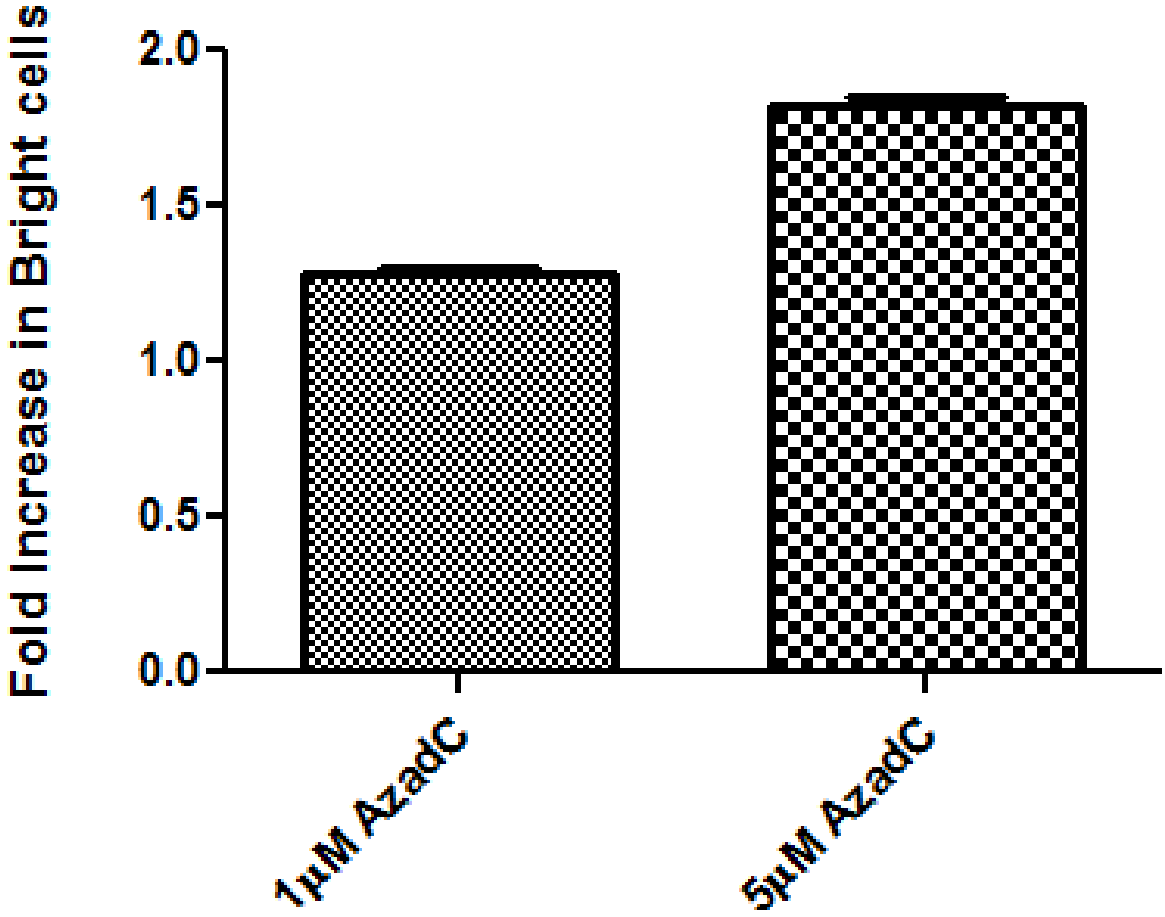


Figure 9. The effect of AzadC concentration on GFP expression class populations.

Cells were induced with dox for 24 hours and then given a daily dose of the indicated concentration of AzadC for 2 days. After a 48-hour recovery, the percentage of Bright cells was determined using FACS. The fold increase in the percentage of bright cells is shown.

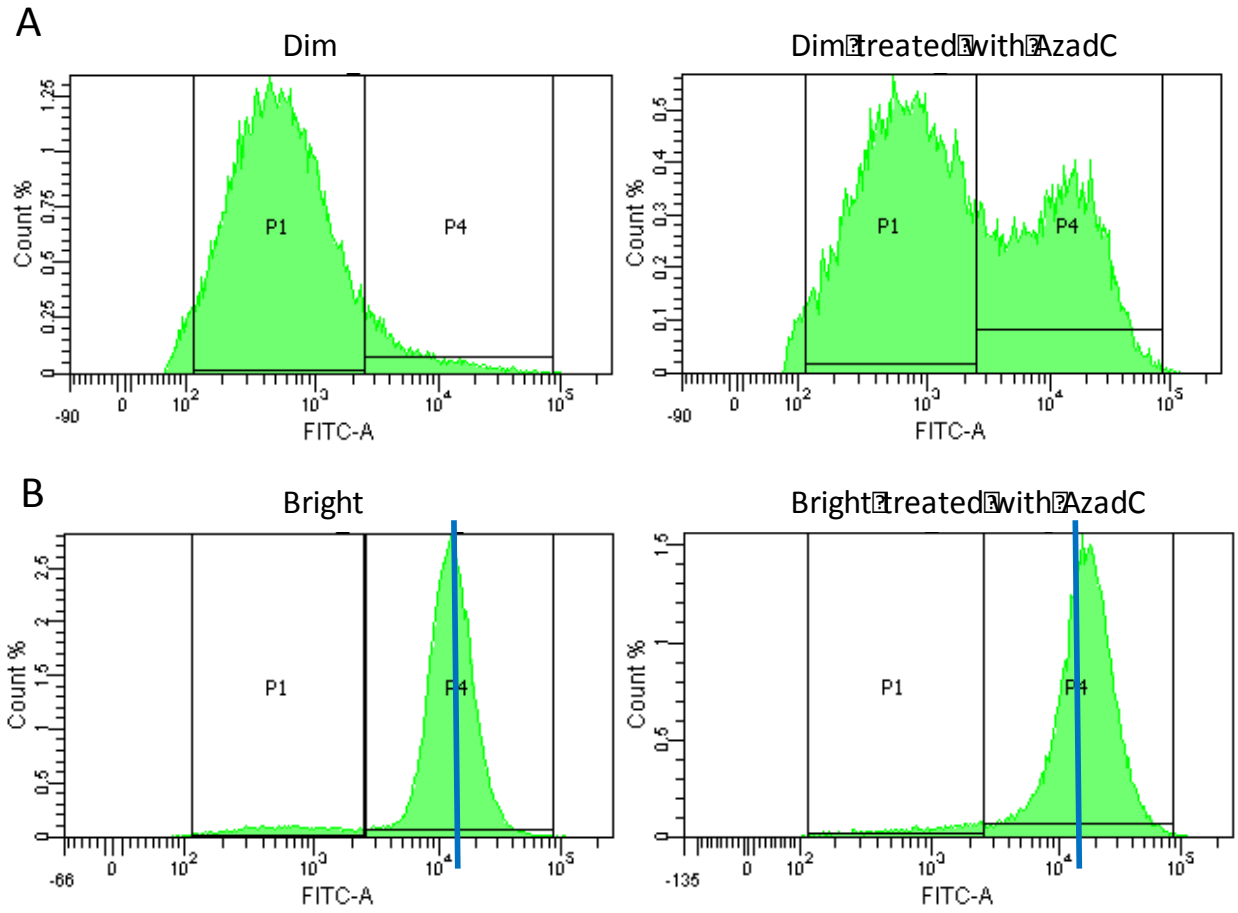


Figure 10. Treatment of sorted populations with AzadC.

Dim (A) and Bright (B) populations were sorted using FACS and then the sorted populations were treated with a daily dose of AzadC for 48 hours. The level of GFP expression was measured using FACS. The blue line in (B) indicates the median of the Bright peak.

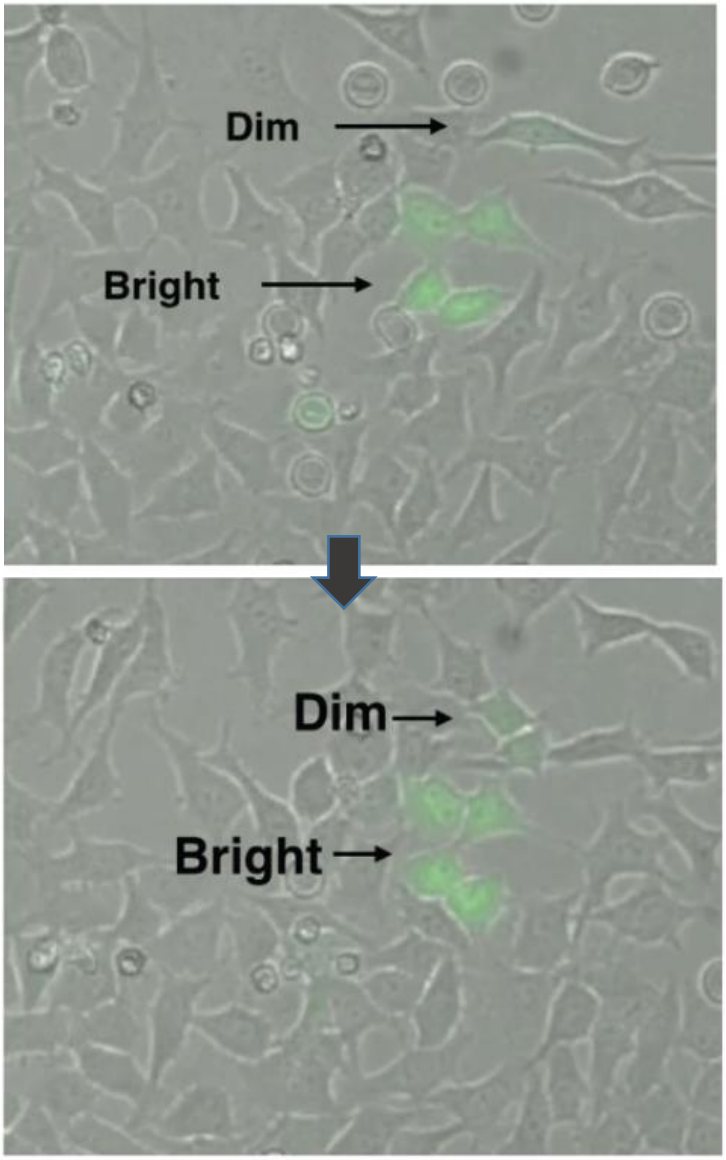


Figure 11. Live-cell imaging of Dim and Bright cells.

Live-cell imaging was used to observe the origination and propagation of cells with Dim and Bright expression of GFP during the 72 hours following a 24 hour dox induction. The arrow indicates the progression of time.

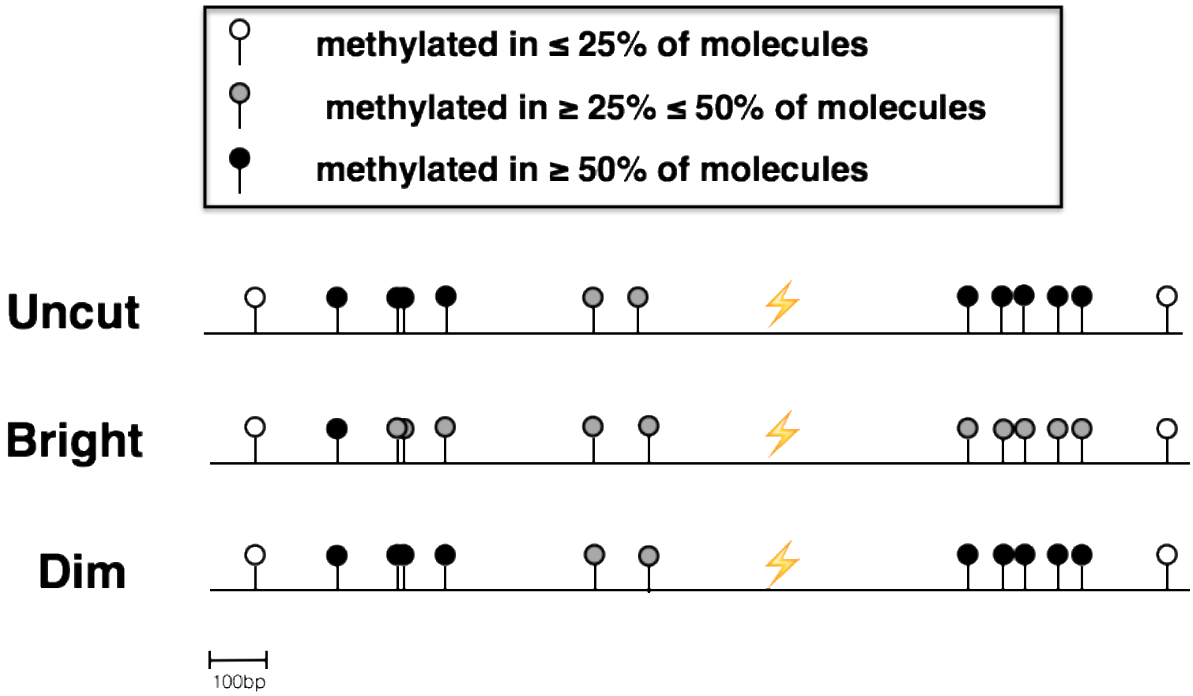


Figure 12. Bisulfite Sequencing of sorted Dim and Bright cells.

Next-Generation Sequencing of bisulfite converted DNA was used to determine the methylation patterns of the area around the site of repair in sorted Dim and Bright cell populations as well as uncut IHN20.22 cells.

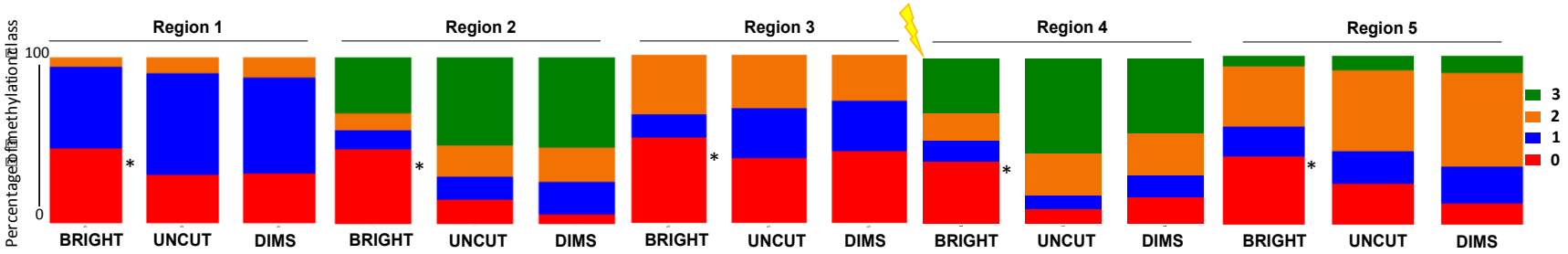


Figure 13. Profile composition of Bright, Dim, and Uncut cell populations.

Epialleles profiles obtained from the analysis of the methylation of each amplicons were subjected to alpha and beta diversity (Qiime). Compositions are grouped by number of methylated CpGs for the five regions adjacent to the DSB. "0 (red color = un-methylated)", "1 (blue = mono-methylated)", "2 (orange = di-methylated)" and "3 (green = tri-methylated)" represent the percent to class of methylation.

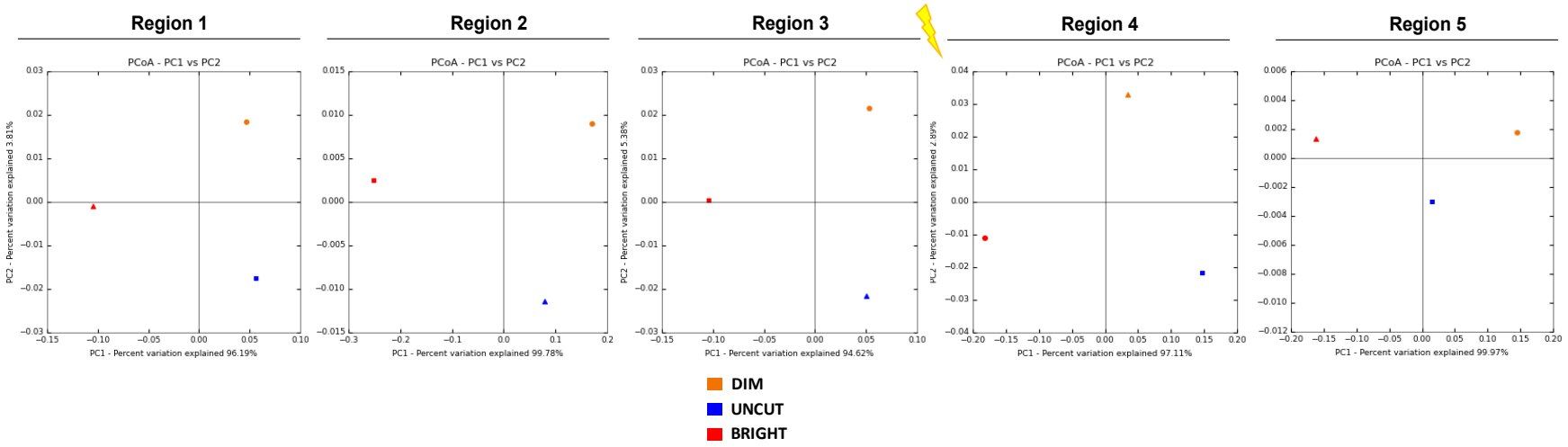


Figure 14. Principal coordinate analysis of Bright, Dim and Uncut populations.

Epialleles profiles obtained from the analysis of the methylation of each amplicons were subjected to alpha and beta diversity (Qiime). In the X and Y axes are represented, respectively, the first and the second components (PC1 and PC2) with the amount of variance in the samples explained by these components, included in brackets. The first principle component represents the highest variance, and the total variance of the samples is the cumulative sum of that described by each of the axes.

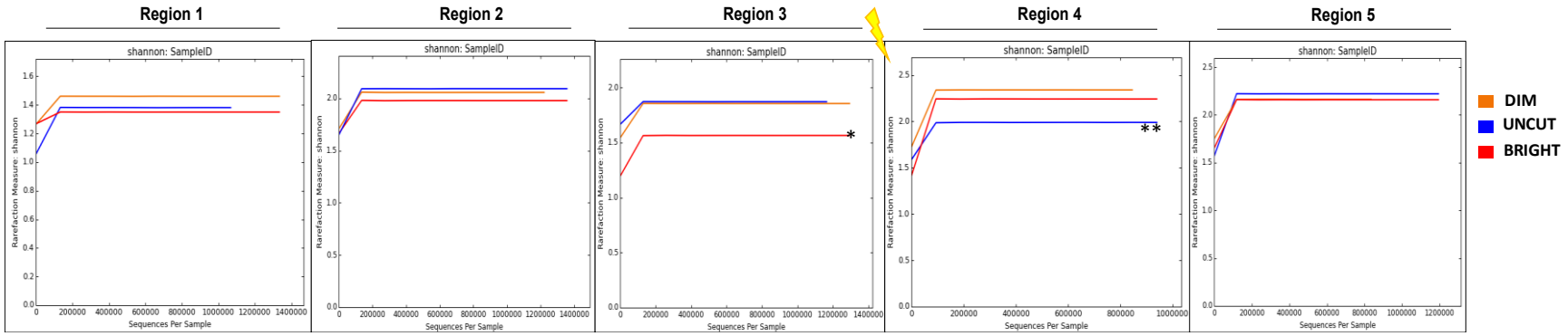


Figure 15. Shannon diversity index between Bright, Dim and Uncut populations.

Epiallele profiles obtained from the analysis of the methylation of each amplicons were subjected to alpha and beta diversity (Qiime). In the X and Y axes are represented, respectively, "number of sequences for sample" and "Rarefaction measure (species richness)" for Shannon Index.

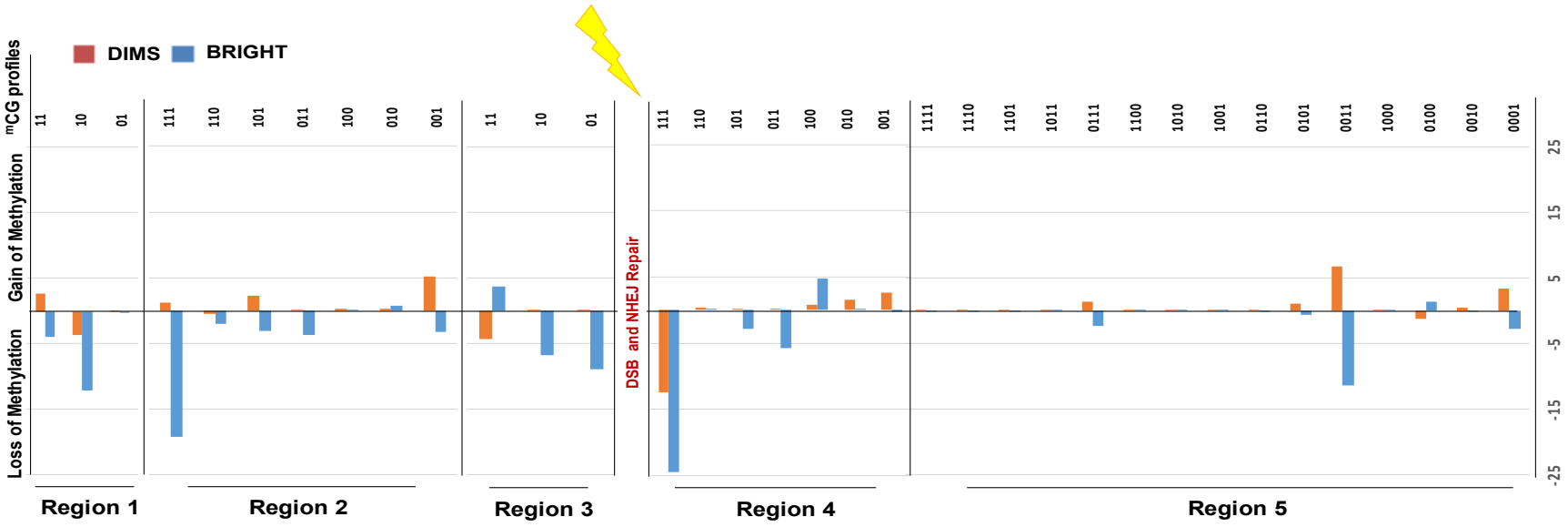
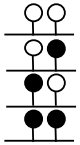


Figure 16. Gain or loss of methylation in Bright and Dim populations compared to the Uncut population.

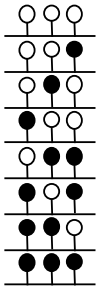
Epialleles profiles obtained from the analysis of the methylation of each amplicons were subjected to alpha and beta diversity (Qiime).

Table 1. Summary table of methylated profiles for region.

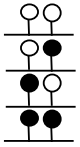
Region 1

profiles	n_meths	UNCUT	DIMS	BRIGHT
	0	29.04	29.88	45.13
	1	59.78	56.10	47.77
	1	1.59	1.70	1.47
	2	9.57	12.30	5.61

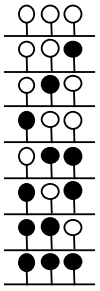
Region 2

profiles	n_meths	UNCUT	DIMS	BRIGHT
	0	14.64	5.85	44.90
	1	12.38	17.59	9.12
	1	0.76	1.02	0.66
	1	0.56	0.81	1.54
	2	9.45	9.57	1.86
	2	5.52	7.83	2.44
	2	3.80	3.27	5.82
	3	52.84	54.01	33.63

Region 3

profiles	n_meths	UNCUT	DIMS	BRIGHT
	0	38.61	42.70	50.67
	1	16.01	16.07	6.85
	1	13.67	13.85	7.14
	2	31.69	27.36	35.32

Region 4

profiles	n_meths	UNCUT	DIMS	BRIGHT
	0	9.14	16.01	37.60
	1	2.22	3.77	3.55
	1	2.20	3.04	2.23
	1	3.79	6.37	6.91
	2	17.86	17.88	12.04
	2	2.76	3.12	1.83
	2	4.60	4.86	2.95
	3	57.39	44.90	32.85

Region 5

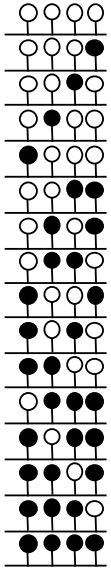
profiles	n_meths	UNCUT	DIMS	BRIGHT
	0	24.15	12.45	40.45
	1	2.86	1.52	4.194
	1	0	0	0.02
	1	5.76	6.21	5.54
	1	10.68	14.05	7.93
	2	0	0	0
	2	0.91	1	0.76
	2	0	0	0
	2	1.96	2.95	1.26
	2	0	0	0
	2	45.06	51.75	33.58
	3	0	0	0
	3	0	0	0
	3	8.56	9.96	6.18
	3	0	0.03	0.01
	4	0	0.02	0

Table 2. List of bisulfite sequencing primers

REGION	PRIMER	SEQUENCE
1	F'	5'-AGGTTAGTTTGGGTTATATGAGAGTTTG-3'
1	R'	5'-TTTCAAACTACCCCATATAACATCTAACC-3'
2	F'	5'-TGGTAAGGGATTTTGTAGATTATTGGATTTAG-3'
2	R'	5'-CTACTATACTCACCCATTATTCTAAAAACAC-3'
3	F'	5'-AGGTTGTATTTTTATTTTTTATAGTTAGGTTTGTTTAGG-3'
3	R'	5'-ATCTAAAAATACATTAAAAAATCCTCTTTCCCCTTC-3'
4	F'	5'-GTGATTATGGTTTTGTTTTTTTTTTTTTGGAATTGT-3'
4	R'	5'-CTAACACTCCCTACTTAATAAAAACTCC-3'
5	F'	5'-GGAGTTTTTATTAAGTAGGGAGTGTTAG-3'
5	R'	5'-CCCCATAAAAACCCACAATATTTCAAATC-3'

CHAPTER THREE:

Introduction

Head and Neck Squamous Cell Carcinoma

Head and neck squamous cell carcinomas (HNSCCs) are squamous cell cancers originating in the lip and oral cavity, nasopharynx, oropharynx, hypopharynx, or larynx. The etiologies of HNSCC are divided into two groups corresponding to the two major risk factors for this type of cancer: exposure to alcohol and tobacco and infection with human papillomavirus (HPV). Smoking increase the risk of HNSCC 5 to 25-fold (72) and the risk is even higher when habitual smoking and alcohol use are combined. Over the last decade, there has been a decrease in smoking- related HNSCCS that is correlated with a decrease in the use of tobacco products. This decline is occurring congruently with an increase in HPV-positive HNSCC incidence, particularly in younger individuals (72-75). The differing causations of HPV-positive and HPV-negative HNSCC essentially produces two diseases with two distinct pathogeneses. In addition to having different biological and clinical phenotypes, HNSCCs that are HPV-positive are associated with a better prognosis than those that are HPV-negative (76,77).

The role of DNA methylation in HNSCC

As discussed in chapter two, changes in DNA methylation patterns are seen across many different type of cancer and HNSCC is no exception. The extent of aberrant methylation patterns in

HNSCC have been recently been shown to have predictive value in determining patient prognosis (78) and is correlated with progression of the disease (79).

While both HPV-negative and HPV-positive HNSCCs exhibit changes in DNA methylation patterns, studies have shown that methylation patterns are different between the two both in a global and site-specific manner (76,80-83). HPV-positive tumors tend to have greater levels or CpG methylation while HPV-negative tumors tend to exhibit global hypomethylation (76,80-82,84). It has been shown that HPV viral oncoproteins can cause changes in DNA methylation and upregulation of DNMTs (85,86) and may interact with DNMTs (87) or be involved in the upregulation of DNMTs by interacting with other regulators like p53 (88,89) and RB1 (90). While this could explain some of the hypermethylation seen in HPV-positive tumors, other mechanisms contributing to hypomethylation and to methylation changes in HPV-negative cancers have not yet been identified.

Circulating oncogenic agents in serum

Studies have also demonstrated the ability of serum from cancer patients to generate tumorigenic phenotypes in treated cells in culture (91-93). This can occur by horizontal gene transfer from circulating, cell-free DNA (93,94) or by the uptake of extracellular vesicles (EVs) that are released into circulation by cancer cells (95-98). Extracellular vesicles, or exosomes, are membrane bound vesicles that may contain membrane or cytosolic proteins, lipids, or nucleic acids for use in intercellular signaling (99). Released by both healthy and cancer cells, exosomes are found in many different body fluids including urine, breast milk blood serum and plasma, amniotic fluid, ascites, semen, and saliva (95,99). Cancer cells use this mechanism to package and deliver oncogenic

proteins (100,101), mRNA and miRNA (100,102,103), or DNA (96) that are capable of causing malignant transformation of recipient cells or facilitate cancer progression and metastasis (95).

Because serum circulates throughout the body and is easily accessible in a clinical setting, it is often examined for the presence of biomarkers that are associated with various disease states. Differing protein (104-107) and RNA(108,109) profiles between healthy individuals and patients with different types and stages of cancer have been identified and found to have diagnostic or prognostic value (110). In addition, epigenetic changes, such as abnormal methylation patterns, have been identified in cancer-associated DNA from blood serum from cancer patients and have been used as biomarkers for detection, diagnosis, and prognosis of many different types of cancer (111-113).

Circulating blood serum may contain a mixture of DNA, RNA including miRNA, and protein, all of which may participate in epigenetic processes. In this work, we investigate whether circulating oncogenic materials in the blood serum of cancer patients, in addition to being useful as diagnostic tools, may contribute to tumorigenesis and progression by causing changes in the normal patterns of epigenetic regulation in distal cells, specifically by altering DNA methylation patterns.

Materials and Methods

Generation of the Dim HeLa cell line

Following induction of DNA damage and repair, NHEJ reporter HeLa cells were sorted based on their fluorescence intensity into Dim and Bright populations (114). Hypermethylation of the Dim cells was confirmed using bisulfite sequencing of an area of the integrated GFP gene.

Human blood serum collection

Serum from 71 HNSCC patients aged between 40 and 88 (Mean age 62 years, STDEV= 9.2) with varying tumor grades and locations in the oral cavity, oropharynx, and larynx were used in this study along with serum from 10 samples from healthy patients aged between 46 and 59. Serum was collected prepared in the Department of Head and Neck Surgery, Greater Poland Cancer Center before surgical treatment. The Institutional Review Board of University of Medical Sciences in Poznan approved the study, and informed consents were obtained from all patients. Blood samples were collected in BD Vacutainer Serum Separation Tubes, incubated for 15 minutes at room temperature to allow coagulation, and centrifuged at 1300 g for 10 minutes. The serum supernatant was transferred to new tubes, centrifuged at 16,000 g for 15 minutes to remove any residual cells and debris, and stored at −80°C (115).

Cell culture

HeLa cells were grown in RPMI basal medium with L-glutamine and supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were grown at 37°C at 5% CO_2.

HNSCC serum treatments

HeLa cells were plated in 96-well plates (Corning) at low density and allowed to attach for several hours. Medium was then removed and replaced with freshly prepared medium containing

10% human serum and no FBS. Cells were allowed to grow for 72 hours before lifting for FACS analysis.

AzadC treatment

Fresh AzadC was prepared by dissolving in DMSO at a concentration of 10mM. HeLa cells were plated in 96-well plates at low density and allowed to attach for several hours. Cells were given a daily dose of AzadC at a final concentration of 1 μM for 48 hours and then lifted for FACS analysis.

FACS analysis

Dim cells were trypsinized and centrifuged at 1000xg for 5 minutes and then re-suspended in PBS at a density of 10^6 cells/mL Cells were run on an Accuri Flow Cytometer. Live cells were selected using a plot of SSC-A vs FSC-A and then a histogram of FL1-A was used to identify and gate cells in the Dim population. Gating and statistical analysis was done using FlowLogic software.

Exosome depletion

Media was prepared using 10% human serum from either HNSCC patients or healthy individuals and placed in 1.5mL ultracentrifuge tubes (Beckman Coulter) and spun at 120,000 x g in a fixed angle rotor at 4°C using a Beckman TLX 120 tabletop ultracentrifuge for 4 hours. The supernatant containing media and serum was removed from the pelleted exosomes and then re-mixed together before it was added to the cells.

RNase A digestion

Media was prepared using 10% human serum from either HNSCC patients or healthy individuals and then digested with 100 μg/mL RNase A (Thermoscientific) for 1 hour at 37°C before it was added to the cells.

Proteinase K digestion

Human serum was digested in 200ng/μL Proteinase K (Promega) overnight at 37°C. Proteinase K was deactivated by incubation in 200μM phenylmethylsulfonyl fluoride (PMSF) (ThermoScientific) before preparing medium with digested serum.

Human serum fractionation

Human serum was filtered using Amicon ® Ultra-0.5mL centrifugal filters. Serum was first filtered through a 30 kDa filter, then the filtrate was filtered through a 10 kDa filter, and then that filtrate was passed through a 5 kDa filter to yield 4 fractions containing different protein size ranges. Media was prepared using 10% of the indicated fraction of serum.

Statistical analysis of fluorescence intensity changes

Mean fluorescence intensity and percent Dim values for cells treated with healthy and caner serum were subjected to a two-tailed t-test with a 95% confidence interval. Graphed results are

indicated as mean ± standard error of the mean (SEM). Statistics and graphs were generated using Prism 5 software (GraphPad).

Results

Circulating hypomethylating agents in HNSCC serum

In order to study the effects of HNSCC patient serum on DNA methylation, we used a GFP reporter cell line that has a methylated GFP gene. This reporter line, which we have named "Dim" was generated during our previous studies where we determined that DNA methylation changes occur following repair by NHEJ (114). Since we have confirmed that the GFP gene in these cells is methylated and that hypomethylation causes an increase in their GFP expression, we can use these cells as reporters for DNA hypomethylating agents.

The Dim HeLa cell line express GFP moderately, appearing as a uniform peak on FACS histograms (Figure 17). Treatment with serum from HNSCC patients causes a populations of Dim cells to increase their expression of GFP. This is visualized on FACS histograms by a shift of cells from the gated "Dim" population into a "Bright" expression class (Figure 18). Treatment with serum from a control group of healthy individuals did not produce the same response (Figure 19). In order to generate a standard for comparison of what FACS histograms of the Dim cells look like when GFP becomes hypomethylated, the cells were treated with, 5-Aza-2'-deoxycytidine (AzadC), a drug with known hypomethylating actions that have led to its approval by the FDA for treatment of myelodysplastic syndrome (24). The shift from Dim to Bright cells following AzadC treatment (Figure 10, A) is unique to the hypomethylating drug and is not seen following treatment with other

types of drugs or sera (data not shown). The similarity in responses of Dim cells to HNSCC patient serum and to the hypomethylating drug indicate that there is some factor in HNSCC patient serum that also acts as a hypomethylating agent.

Unlike their response to normal human serum, the response of Dim cells to serum from different HNSCC patients was heterogeneous. Serum from some patients caused a significant shift from the Dim to the Bright GFP expression class (Figure 18) while the effect of serum from other patients was not as pronounced (Figure 20). Serum that caused greater shifts were designated as "Responders" while those effecting only small shifts were classified as "Partial Responders". Still others had little to no effect on GFP expression and were termed "Non-Responders" (Figure 21). There was a positive correlation of the expression level of GFP determined by qPCR and the fluorescence intensity measured by FACS ($r=0.37$, $p=0.04$), indicating that the shift seen in FACS data was in fact due to changes in expression of the GFP gene. Although there was diversity in the response of Dim cells to the serum from different HNSCC patients, the difference in their overall response to serum from cancer serum versus healthy human serum was statistically significant (Figure 22). The percent of the cells contained in the Dim category was decreased and the mean fluorescence intensity was increased in cells treated with cancer serum compared to those treated with healthy human serum. Thus, serum from HNSCC cancer patients, but not from healthy individuals, has the capacity to induce hypomethylation in treated cells. Further, continuous serum exposure was required to maintain the GFP expression change. When cells were treated with human serum and then allowed to recover in normal culture medium containing FBS, the cells returned to their original expression class (Figure 23).

Identifying the type of hypomethylating agent in HNSCC serum

Although it has been demonstrated that exposure to HNSCC patient serum alters methylation profiles in treated cells compared to normal human serum, it is still unclear what elements are contained in the serum that are causing these effects. In order to identify the causative agent, we used a knockout method by removing specific types of serum components individually and then using serums classified as "Responders" as a screen to measure the ability of the treated serum to generate the same hypomethylation response. We first hypothesized that miRNA contained in the serum may be responsible. To investigate this, we pre-treated serum with RNase A before treating the cells. This treatment had no effect on the serums' hypomethylating actions (Figure 24, Figure 25). Since the RNA contained in serum is known to be contained in exosomes (95) which would prevent their exposure to the RNase A treatment, we next depleted the serum of exosomes prior to cell treatment. As with RNase A treatment, exosome depletion did not hinder the hypomethylating effects of HNSCC patient serum (Figure 26, Figure 27).

Next, we sought to determine if the source of the methylation changes was a protein, so we digested the serum with Proteinase K prior to treating the cells. Removal of protein from the serum ablated the shift from low GFP expression to high GFP expression that was previously observed when Dim cells were treated with HNSCC patient serum (Figure 28). Since the difference in mean fluorescence intensity between HNSCC serum treated Dim cells with and without Proteinase K treatment is statistically significant (Figure 29), these data identify the hypomethylating effector as a protein contained only in HNSCC serum or in different quantities than normal human serum.

To obtain more information about what protein is causing the hypomethylating effects of HNSCC patient serum, we filtered the serum into 4 fractions based on protein size so that each

fraction contains proteins within a specific size range. Treatment of Dim cells with the fraction containing proteins that are greater than 30 kDa caused a shift in GFP expression that appears even more pronounced than that caused by the total serum, while fractions containing only smaller proteins produced little or no change in GFP expression (Figure 30). These results classify the unknown protein as being greater than 30 kDa. Since 10% serum was used in each treatment, the fractions contained concentrated amounts of proteins in their respective size range. The increased concentration of the hypomethylating protein in the greater than 30 kDa fraction caused more hypomethylation in Dim cells than the total serum with all proteins.

To clarify if the epigenetically active protein was also contained in healthy human serum but in smaller amounts, we filtered the serum from a healthy individual into two fractions, one containing proteins greater than 30 kDa and one containing proteins smaller than 30 kDa. If the protein is also contained in this serum, we would expect that concentration of the proteins that are over 30 kDa would concentrate this protein enough to produce a change in GFP expression of the dim cells. Neither fractions from this serum produced a shift in GFP expression in Dim cells (Figure 31), confirming that the hypomethylating protein is present only in the serum from the cancer patients.

Discussion

Cancer cells may induce epigenetic changes in distant cells

The importance of epigenetic changes in cancer progression has already been discussed, but the potential for cancer cells to broadcast epigenetic changes to cells in other areas of the body has

critical implications on their metastatic potential. This gives cancer cells the power to incite carcinogenic processes in distant, healthy cells and facilitate progression of the disease. Further, although the model presented in this paper reports that HNSCC patient serum can cause hypomethylation in exposed cells, it does not exclude the possibility of other factors in serum that may affect epigenetic regulation in other ways such as DNA hypermethylation or changes in histone marks.

Studies have suggested a correlation between cancer stage and serum levels of epigenetic regulators like DNMT1 and HDAC1 (116,117), however, to our knowledge, this is the first study demonstrating epigenetic change as a result of circulating, cancer-associated proteins. This finding is especially interesting given our limited knowledge of how DNA demethylation occurs in animal cells despite the fact that, as previously mentioned, hypomethylation frequently contributes to cancer progression by activating oncogenes (8). HNSCCs, specifically, have been shown to have increased levels of global hypomethylation that correlate with smoking and tumor stage (118) and to be more pronounced in HPV-negative tumors (81). In fact, studies have demonstrated a correlation between smoking and DNA hypomethylation independently of HNSCC (119,120).

Mechanisms of DNA demethylation

DNA demethylation may occur passively as a result of a failure of DNMTs to propagate methylation marks to daughter DNA strands during replication, or actively by conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) through the enzymatic activity of the ten-eleven translocation (Tet) family of dioxygenases. 5hmC is then further oxidized by Tet to form 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) which can then be excised and replaced with

cytosine by base excision repair (BER) (121,122). Studies have demonstrated that Tet enzymes are often mutated or downregulated in cancer which can contribute to hypermethylation that is characteristic of cancer cells (123-125). This does not explain the aberrant hypomethylation that is seen throughout the development of cancer and that is highlighted in this study.

Another explanation for progressive demethylation during tumorigenesis is alterations in the structure of chromatin which makes the DNA more susceptible to demethylation. It is widely understood that DNA methylation contributes to the condensation of chromatin and thus local inactivation of genes, however less known are studies that have shown that this process occurs bilaterally. Deacetylation of histones causes changes in chromatin structure to an inactive state that protects DNA from demethylation, while deacetylation opens chromatin and allows for demethylase activity. This is supported by the demonstrated ability of inhibitors of histone deacetylases (HDACs) and histone acetyltransferases (HATs) to cause DNA hypomethylation (121,126-128).

Potential identities of the unknown protein

While the specific identity of the hypomethylating protein discovered in this study remains unresolved, fractionation of the serum by protein size allowed for smaller growth factors and signaling molecules to be eliminated as candidates. While it is possible that the serum contains a protein that is directly responsible for enzymatic demethylation of DNA, it is more likely that it contains a protein that is causing an indirect demethylation effect due to interaction with other cellular components. For example, the protein could affect histone acetylation which could in turn alter DNA methylation patterns. Alternatively, the protein could affect the expression, processing,

or stability (129,130) of miRNAs that are involved in epigenetic mechanisms (131,132) of gene expression or in regulation of epigenetic proteins.

Future studies will be aimed at uncovering the identity of the circulating, cancer-associated hypomethylating protein contained in HNSCC patient serum. In addition to providing a potential target for diagnostics, a biologically active, circulating protein with tumorigenic effects could be an ideal therapeutic target to interrupt the metastatic progression of cancer.

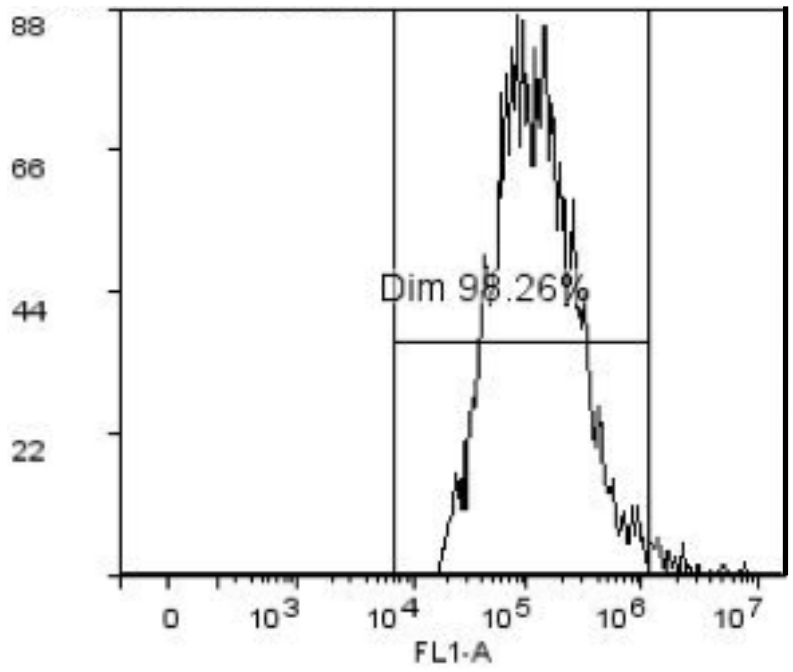


Figure 17. FACS analysis of Dim HeLa cells grown in FBS.

Histogram of GFP expression of the Dim HeLa cell line under normal culture conditions with gated "Dim" population.

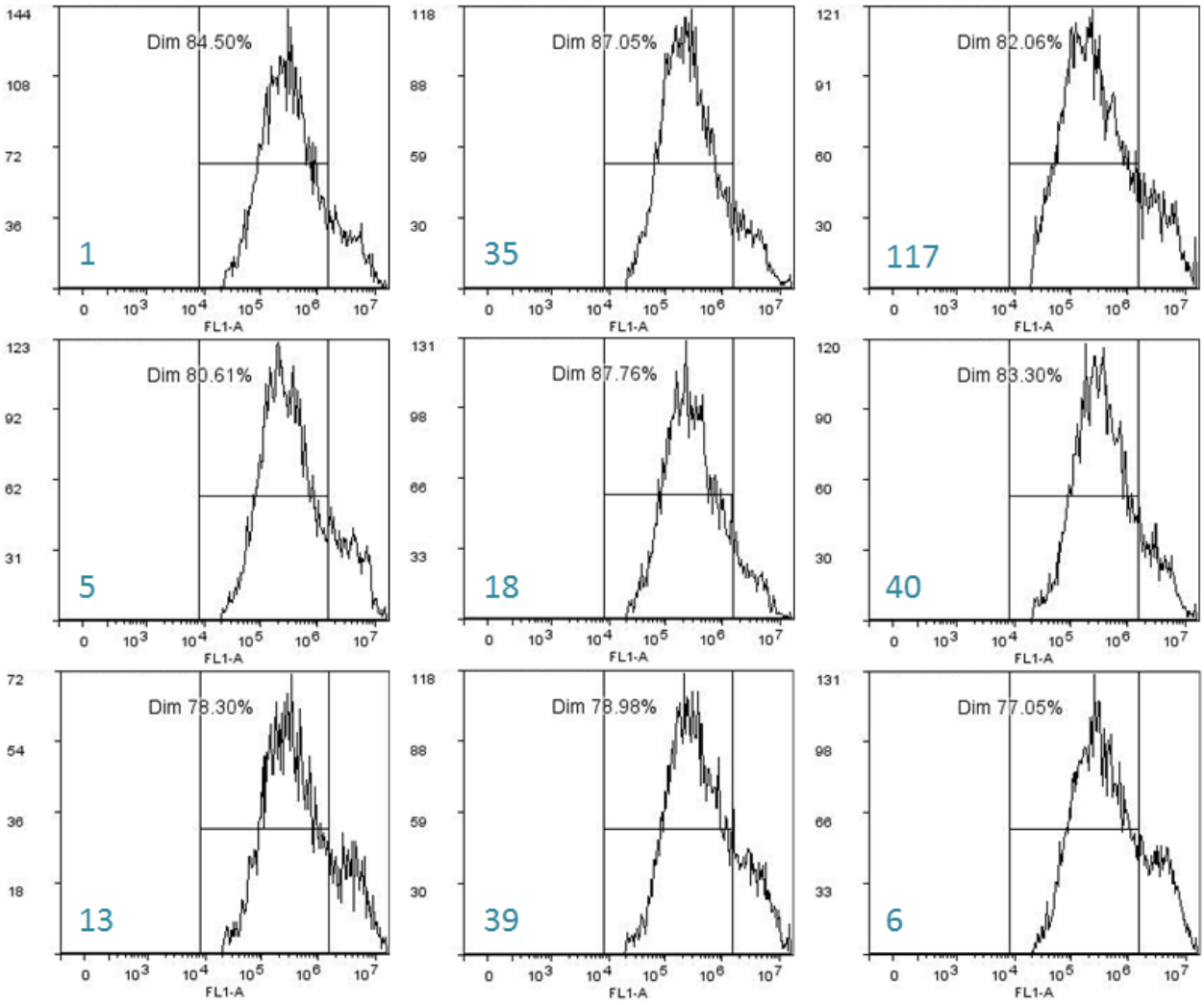


Figure 18. FACS of Dim HeLa cells treated with HNSCC patient serum: Responders.

FACS histograms of the GFP expression levels of Dim HeLa cells after treatment with HNSCC patient serum. Cells in the "Dim" expression are gated as in Figure 17 and numbers indicate the serum sample number. These serums are called "Responders" as they produced the biggest shifts in GFP expression compared to other serums.

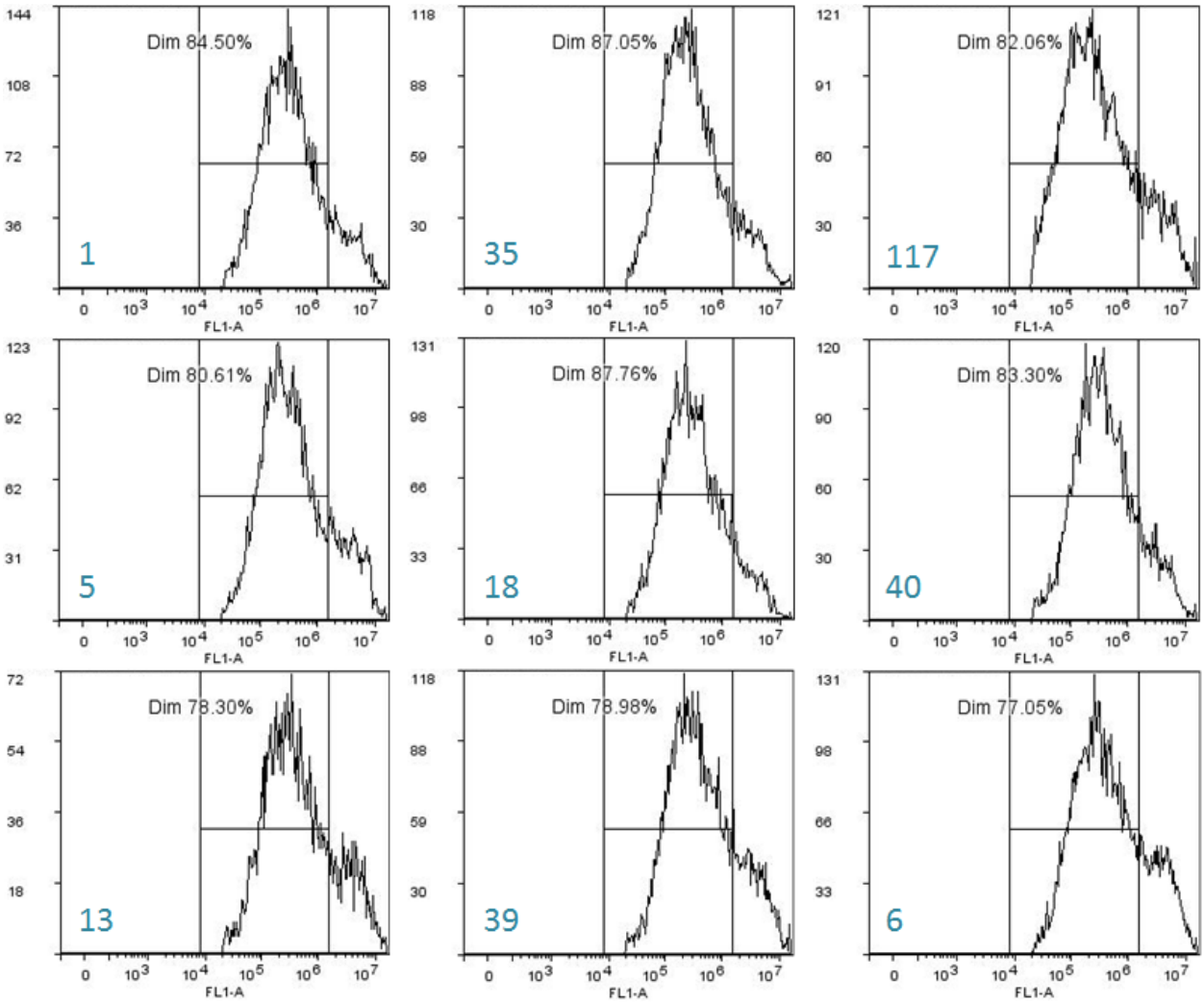


Figure 19. FACS histograms of Dim HeLa cells treated with normal human serum.

FACS histograms of the GFP expression levels of Dim HeLa cells after treatment with healthy human serum. Cells in the "Dim" expression are gated as in Figure 17 and numbers indicate the serum sample number.

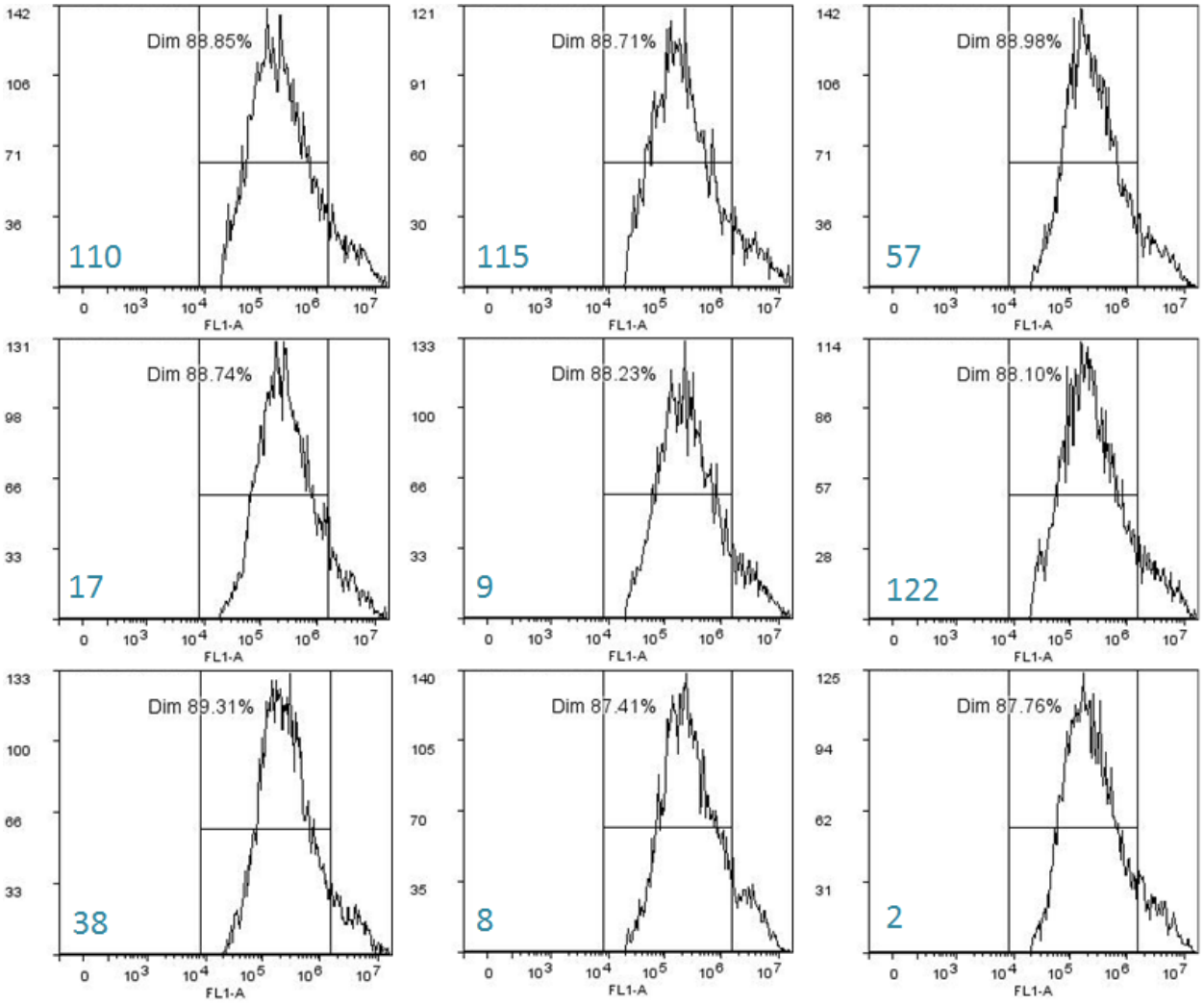


Figure 20. FACS histograms of Dim HeLa cells treated with HNSCC patient serum: Partial Responders.

FACS histograms of the GFP expression levels of Dim HeLa cells after treatment with HNSCC patient serum. Cells in the "Dim" expression are gated as in Figure 17 and numbers indicate the serum sample number. These serums are called "Partial Responders" as they produced a significant shift in GFP expression but not as pronounced as those in the "Responder" category.

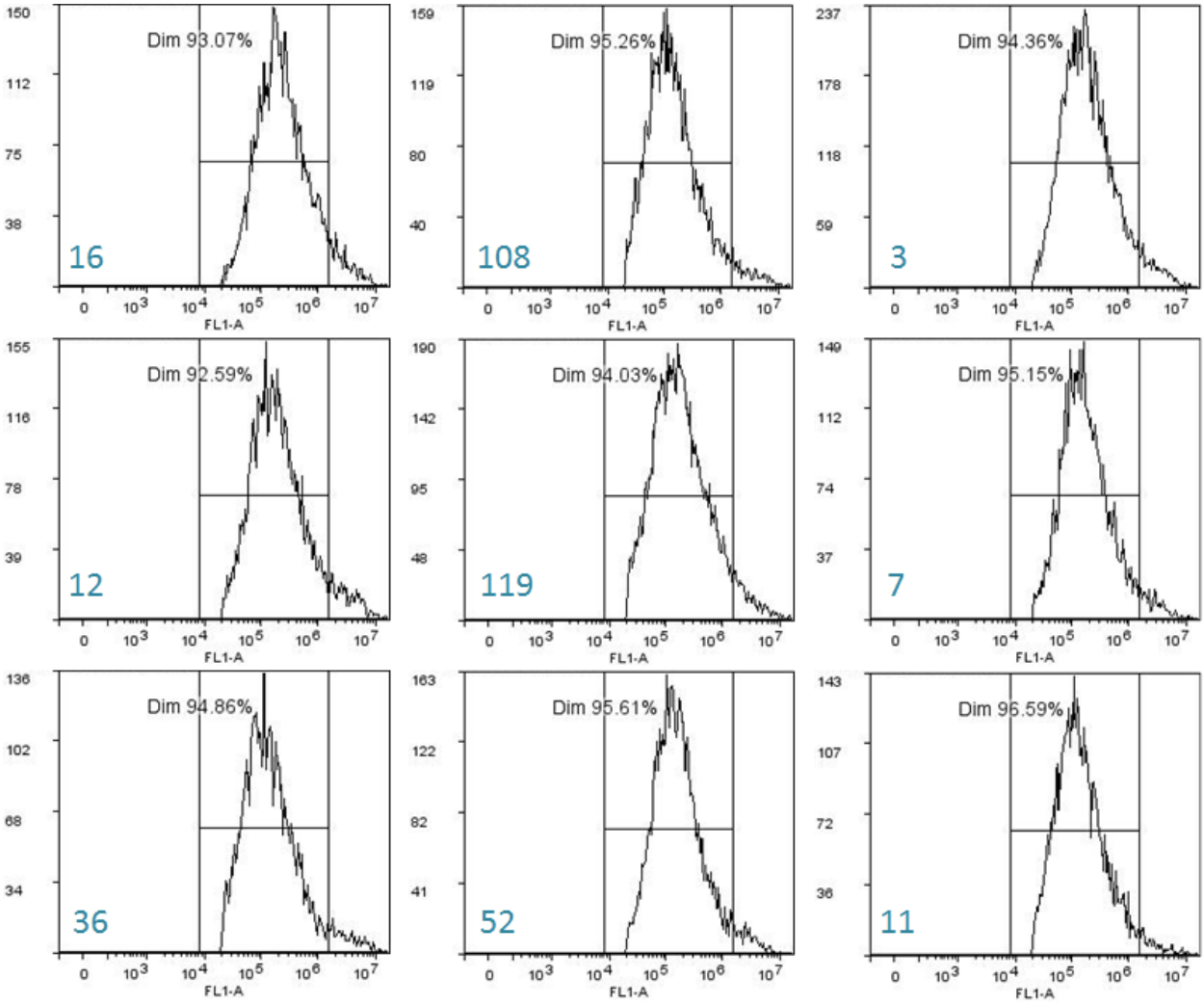


Figure 21. FACS histograms of Dim HeLa cells treated with HNSCC serum: Non-Responders.

FACS histograms of the GFP expression levels of Dim HeLa cells after treatment with HNSCC patient serum. Cells in the "Dim" expression are gated as in Figure 17 and numbers indicate the serum sample number. These serums are called "Non-Responders" as they produced little or no shift in GFP expression.

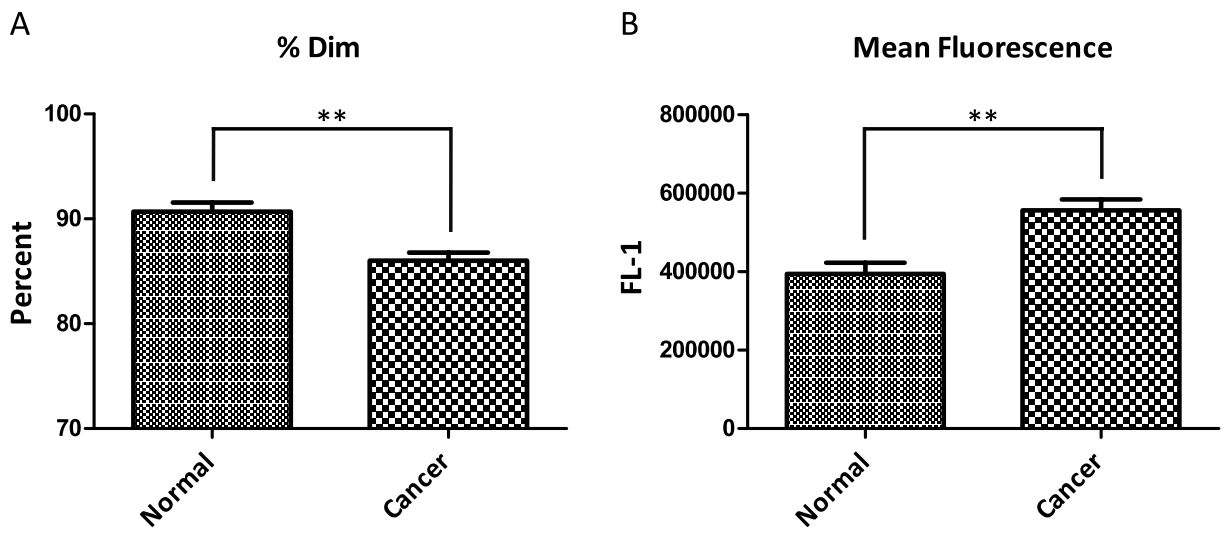


Figure 22. Statistical analysis of the effect of normal human and HNSCC patient serum on GFP expression in Dim HeLa cells.

The percentage of cells in the Dim expression class (A) ($p=0.0015$) and the mean fluorescence intensity (B) ($p=0.0018$) was compared for cells treated with HNSCC patient serum ($n=68$) and normal human serum ($n=10$).

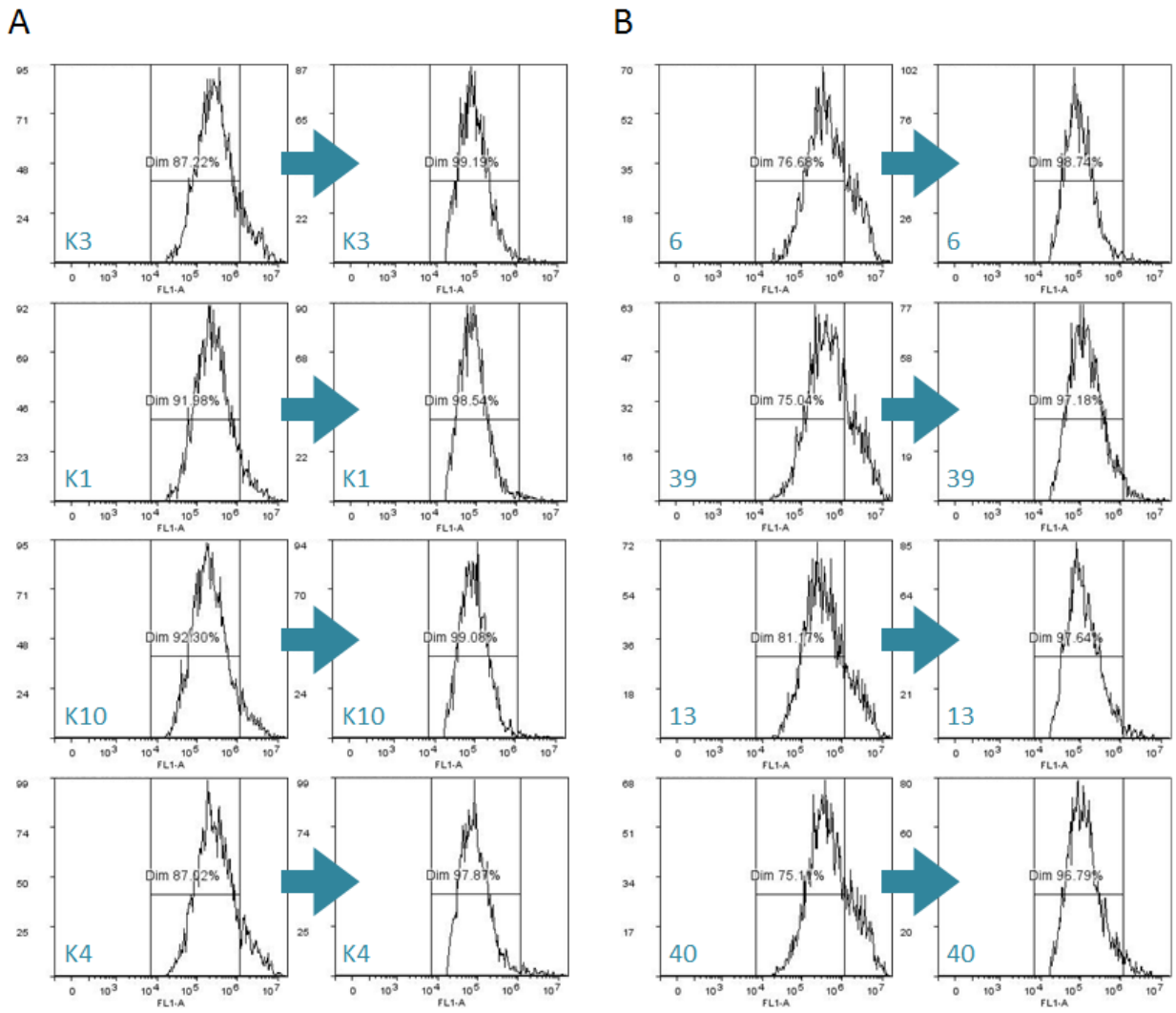


Figure 23. Treatment with human serum followed by recovery in FBS.

FACS histograms of the GFP expression levels of Dim HeLa cells after treatment with normal human serum (A) or HNSCC patient serum (B) for 72 hours (graph to the left of each arrow) followed by recovery in normal media with FBS for 6 days (graph to the right of each arrow). Serum sample number is indicated in the bottom left corner of each graph.

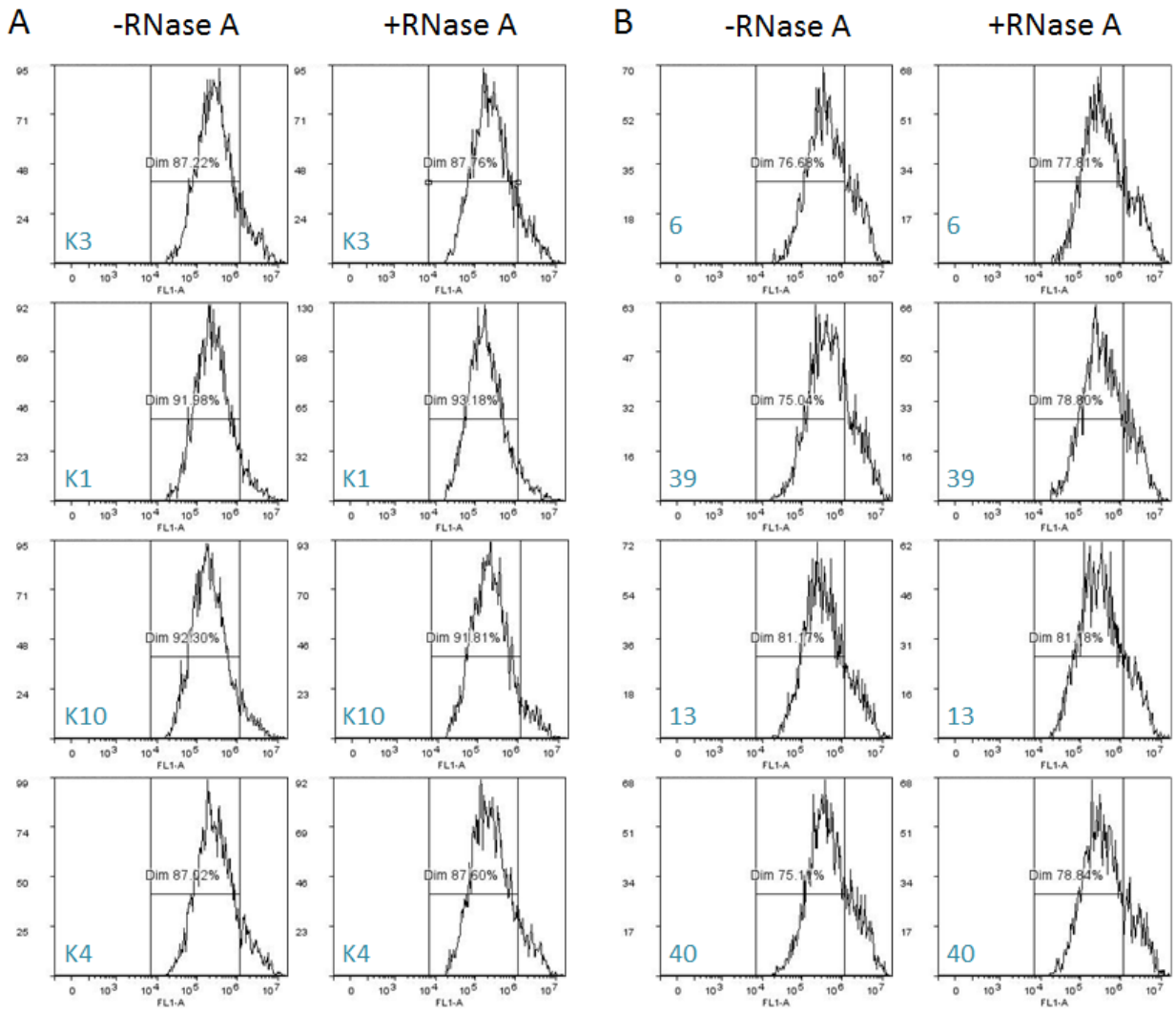


Figure 24. Degradation of RNA in HNSCC serum.

FACS histograms of the GFP expression levels of Dim HeLa cells after treatment with normal human serum (A) or HNSCC patient serum (B) with and without digestion with RNase A. Serum sample number is indicated in the bottom left corner of each graph.

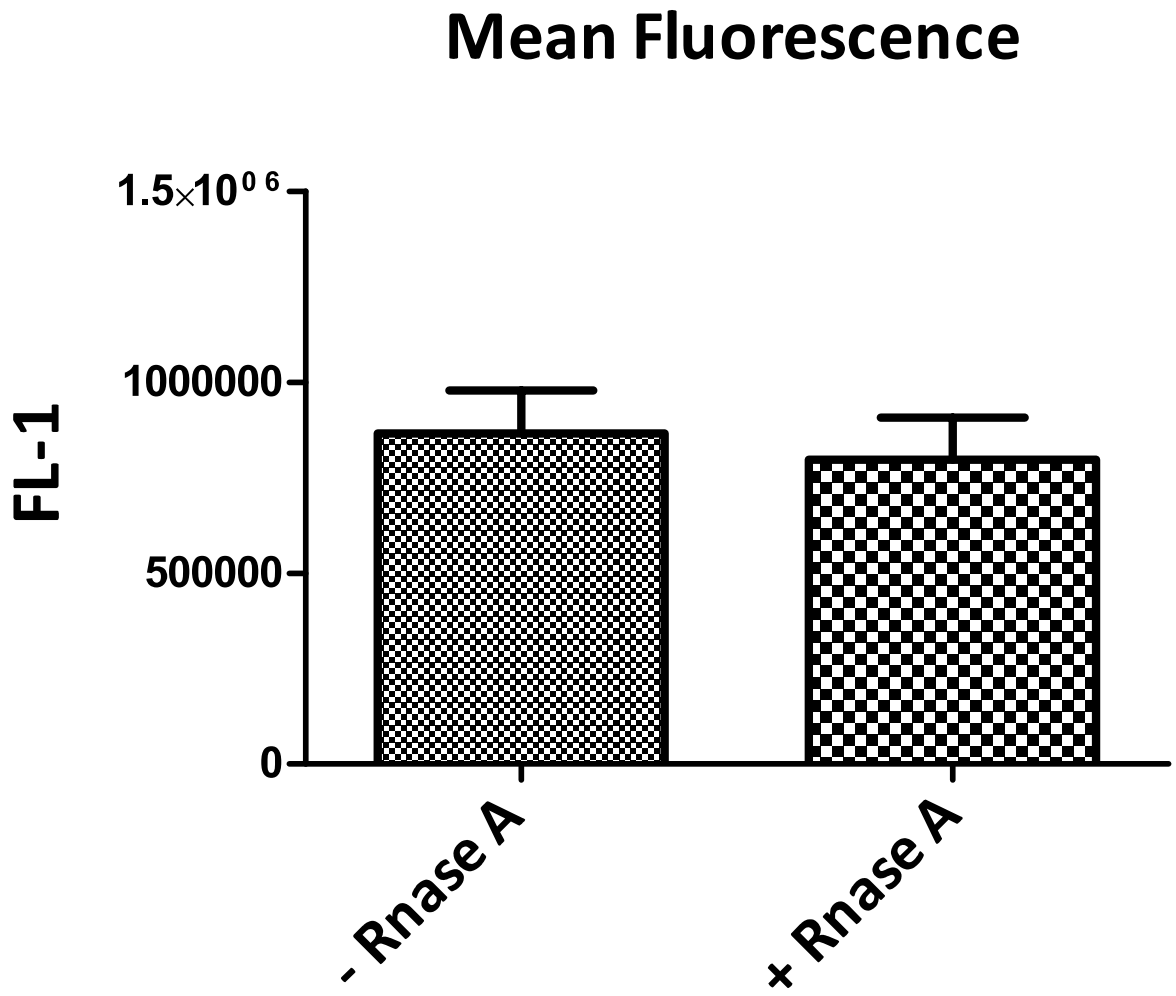


Figure 25. Statistical analysis of the effect of RNA degradation on the hypomethylating effects of HNSCC patient serum.

The mean fluorescence intensity of Dim cells after treatment with serum with or without RNase A digestion.

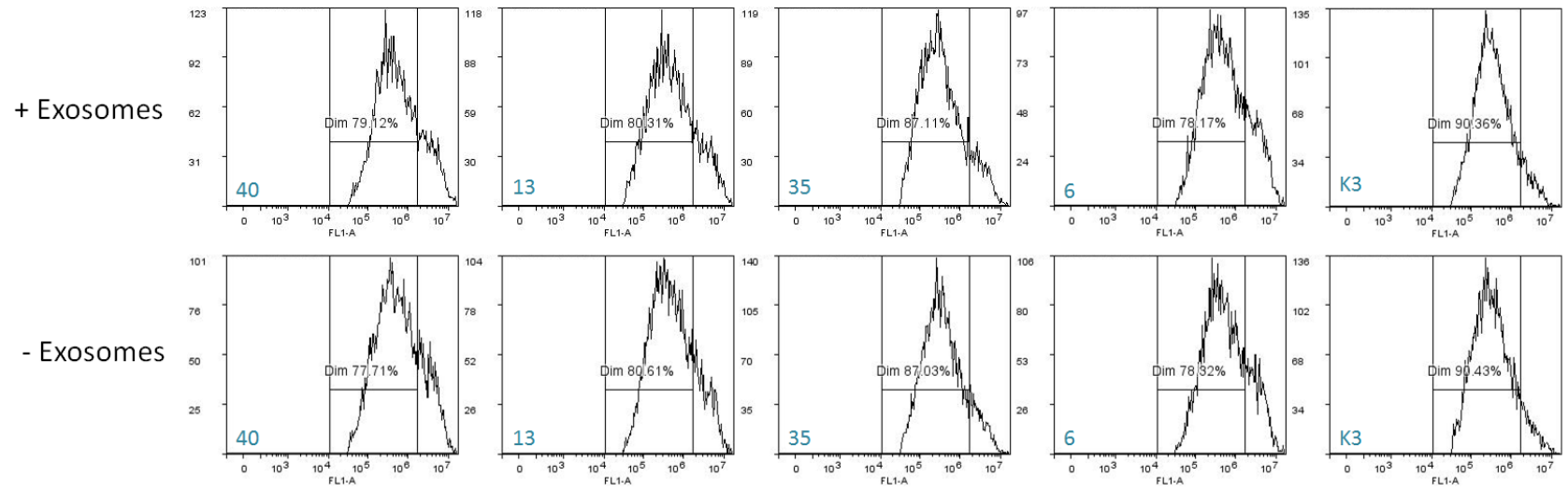


Figure 26. Treatment of Dim HeLa cells with exosome depleted human serum.

FACS histograms of the GFP expression levels of Dim HeLa cells after treatment with normal human serum or HNSCC patient serum with and without exosome depletion. Serum sample number is indicated in the bottom left corner of each graph.

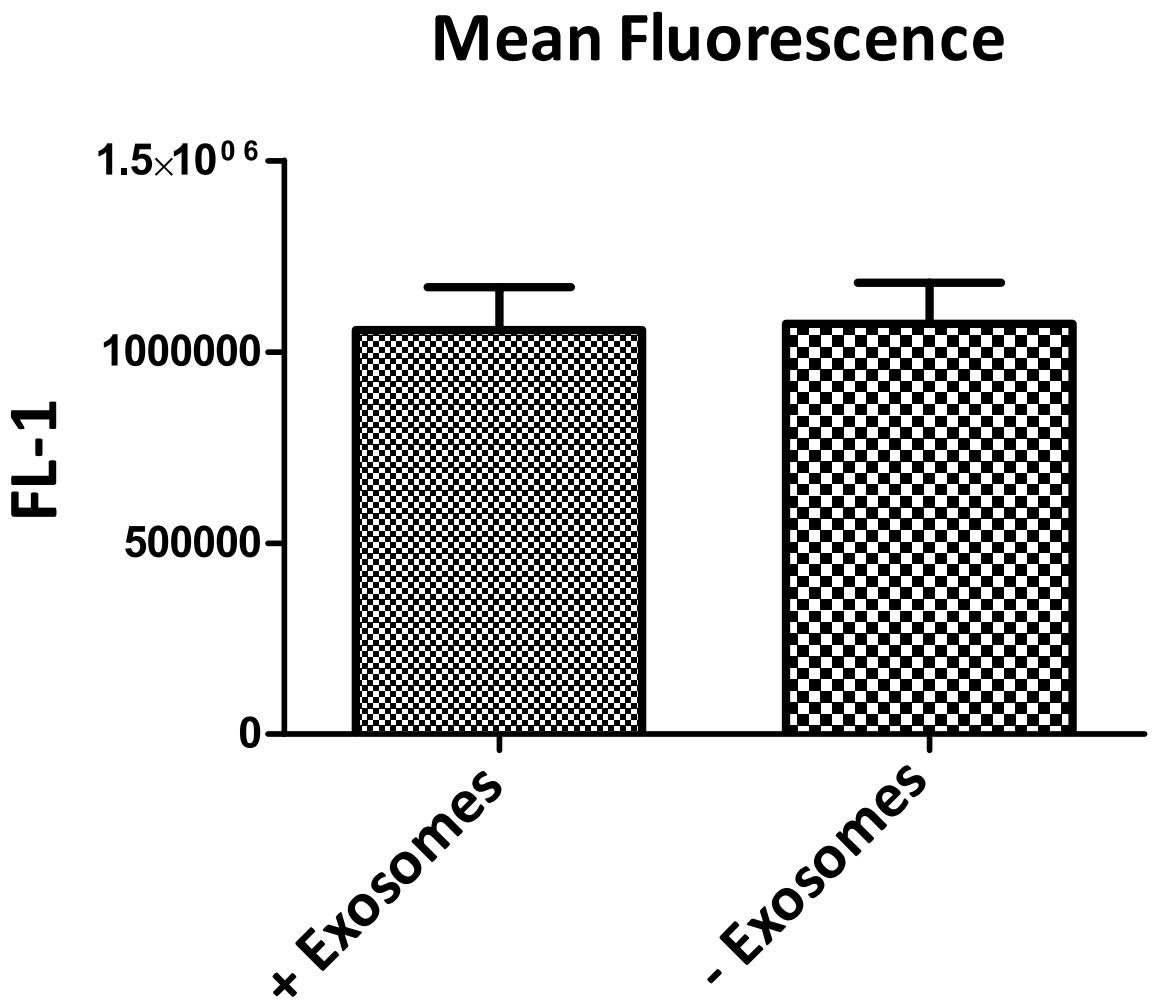


Figure 27. Statistical analysis of the effect of exosome depletion on the hypomethylating effects of HNSCC serum.

The mean fluorescence intensity of Dim cells after treatment with serum with or without exosome depletion.

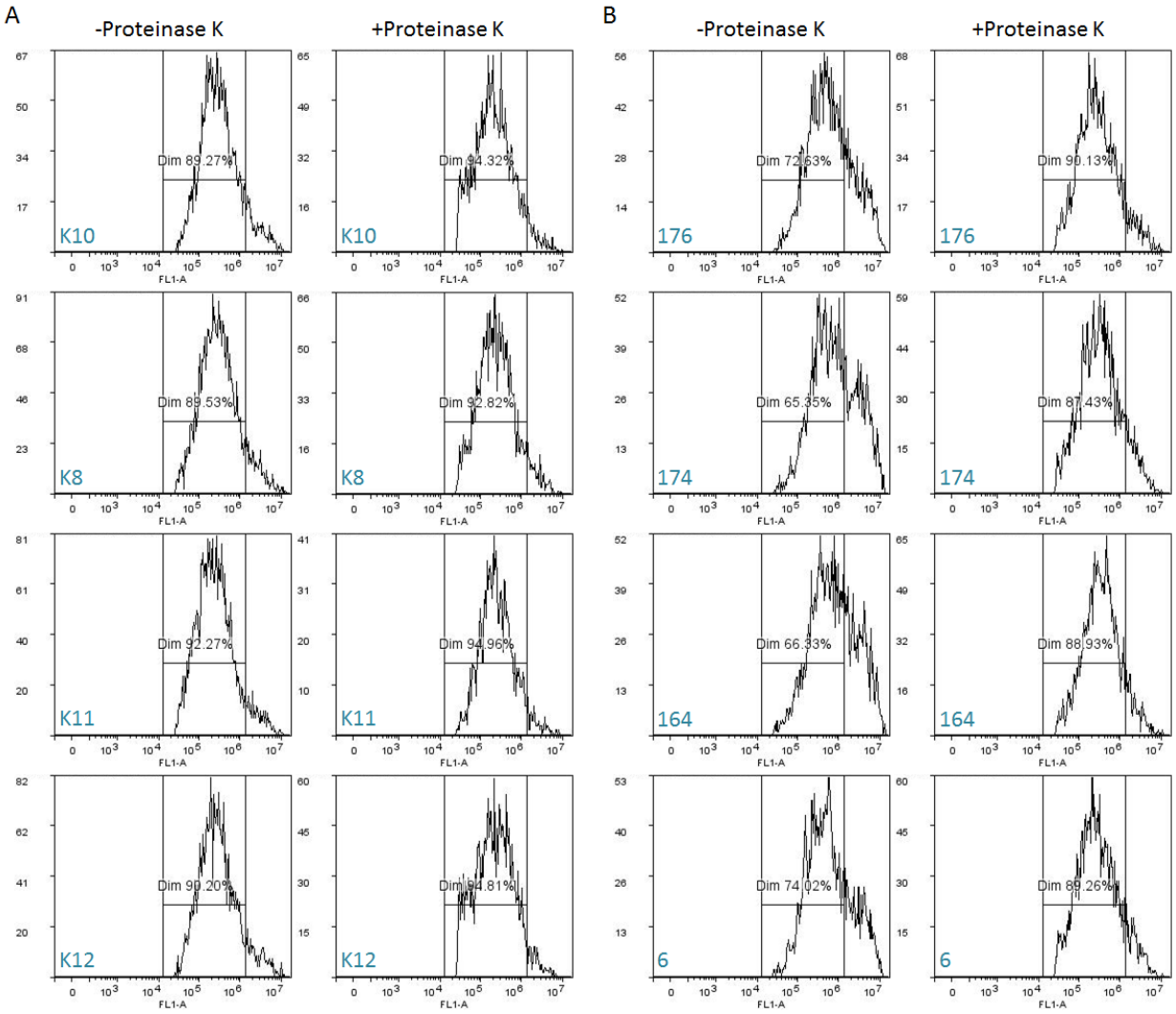


Figure 28. Degradation of protein in human serum.

FACS histograms of the GFP expression levels of Dim HeLa cells after treatment with normal human serum (A) or HNSCC patient serum (B) with and without digestion with Proteinase K. Serum sample number is indicated in the bottom left corner of each graph.

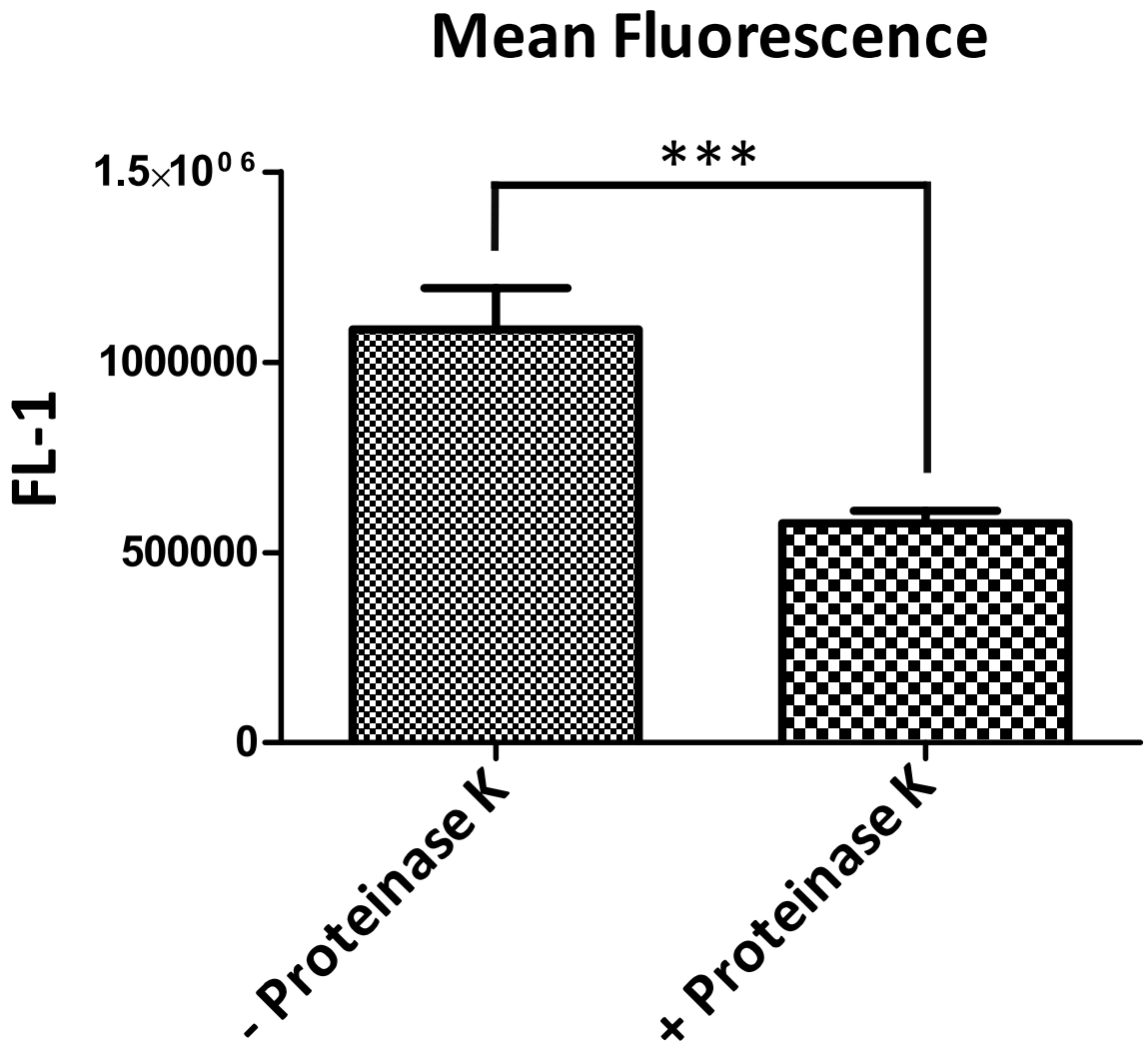


Figure 29. Statistical analysis of the effect of protein degradation on the hypomethylating effects of HNSCC serum.

The mean fluorescence intensity ($p=0.0001$) was compared for cells treated with HNSCC patient serum ($n=15$) with and without Proteinase K treatment.

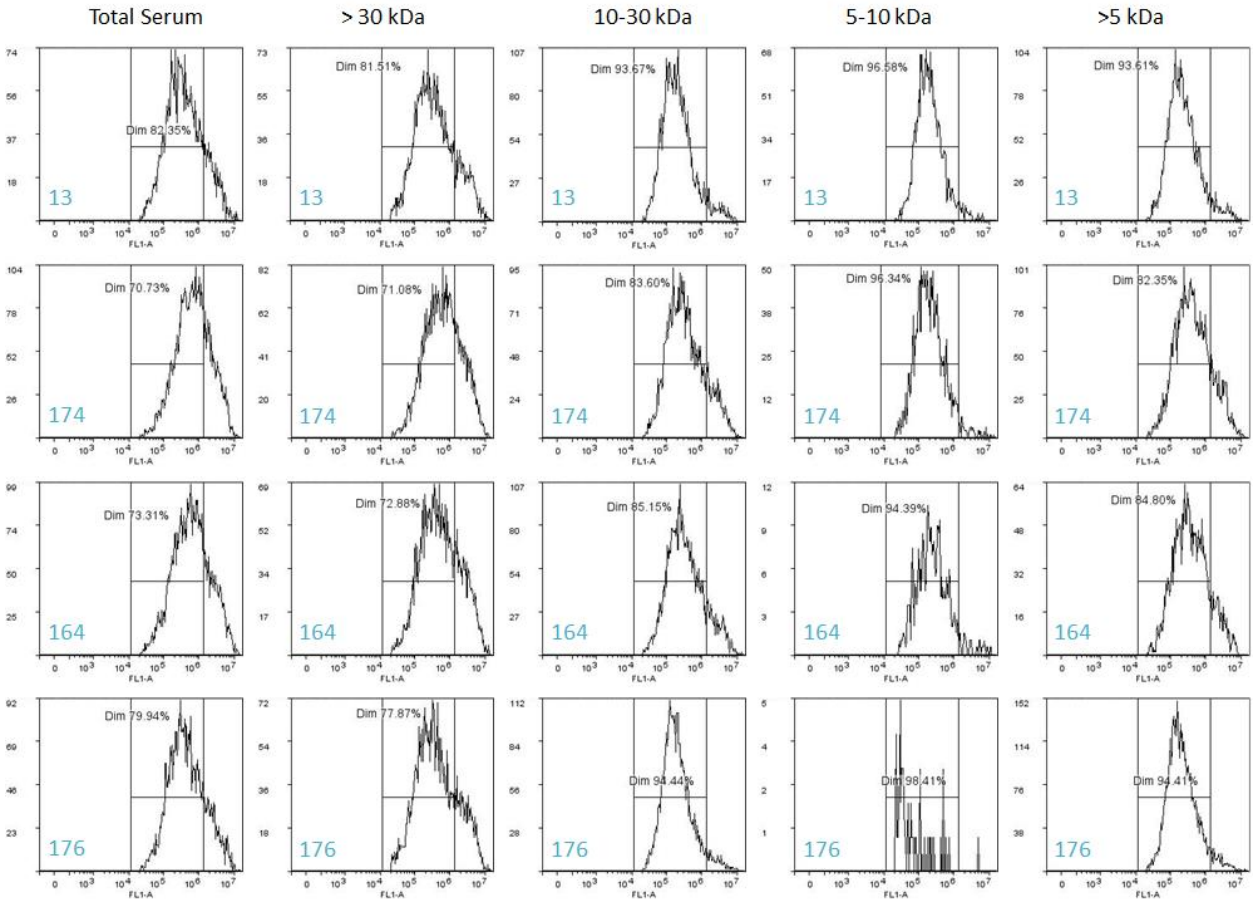


Figure 30. Filtration of HNSCC serum into fractions with differing protein sizes.

FACS histograms of the GFP expression levels of Dim HeLa cells after treatment with total HNSCC patient serum and with serum fractions that were filtered based on protein size. Rows contain data for different serum samples and columns contain data for fractions of different protein size ranges. Serum sample number is indicated in the bottom left corner of each graph and protein size range is indicated at the top of each column.

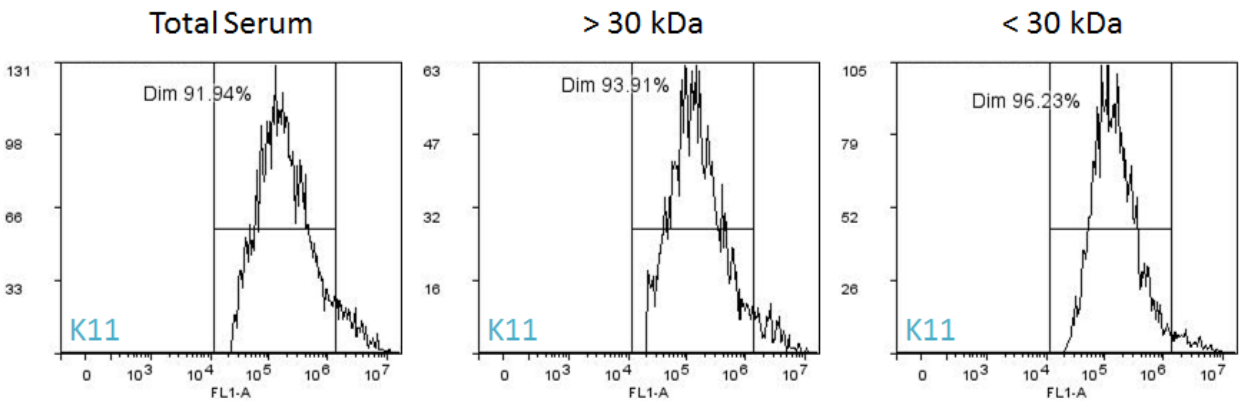


Figure 31. Filtration of healthy human serum into fractions with differing protein sizes.

FACS histograms of the GFP expression levels of Dim HeLa cells after treatment with total healthy human serum and with serum fractions that were filtered based on protein size. Serum sample number is indicated in the bottom left corner of each graph and protein size range is indicated at the top of each graph.

CHAPTER FOUR

Introduction

MiRNAs in cancer

MicroRNAs are small, non-coding RNA molecules that are involved in gene regulation. After being transcribed, primary miRNA (pri-miRNA) transcripts are processed into pre-miRNA hairpin structures before being cleaved into short, dsRNA fragments. Finally, one strand of the fragment is then degraded to form the mature miRNA.

MiRNAs participate in regulation of gene expression by forming RNA-induced silencing complexes (RISCs) that target complementary sequences on mRNA and either inhibit translation or cause degradation. Further, miRNAs can contribute to tumorigenesis either by upregulation of a miRNAs targeting tumor suppressor genes or downregulation of miRNA targeting oncogenes (133). For example, miR124a is frequently down regulated in several cancer types including colon, breast, and lung carcinomas as well as some leukemias and lymphomas. Because this miRNA is a negative regulator of CDK6, down regulation caused by hypermethylation results in increased levels of CDK6 which in turn facilitates inactivation of RB1 via phosphorylation (141). MiRNAs have also been shown to regulate epigenetic processes by targeting DNMTs, and histone methylating EZH2 complexes (142-144). Aberrant miRNA expression can result from chromosomal deletions, mutations affecting genes involved in miRNA processing, or by epigenetic mechanisms that affect miRNA expression (134). DNA methylation and chromatin remodeling processes can cause dysregulation of miRNA in the same way as they do with gene encoding transcripts (135-137) as

evidenced by the ability of HDAC inhibitors (138,139) and hypomethylating drugs (140) to induce changes in miRNA expression. This demonstrates interdependent regulation between the mechanisms.

miRNAs in HNSCC

Studies have demonstrated differing miRNA expression profiles between cancer tissue and adjacent healthy tissue in HNSCC patients (145), between serum from HNSCC patients and serum from healthy individuals (115) and between serum collected from HNSCC patents before and after treatment (146). Further. several of these miRNA have been shown to have diagnostic or prognostic value (147). As we have discussed, blood serum contains circulating factors that can cause malignant transformation in distal cells and we have already demonstrated the ability of HNSCC patient serum to epigenetically alter gene expression (Chapter 3). In this study we investigate the potential for cancer serum to effect miRNA regulation in exposed cells.

Materials and Methods

Human blood serum collection

Serum was collected prepared in the Department of Head and Neck Surgery, Greater Poland Cancer Center before surgical treatment. The Institutional Review Board of University of Medical Sciences in Poznan approved the study, and informed consents were obtained from all patients. Blood samples were collected in BD Vacutainer Serum Separation Tubes, incubated for 15 minutes at room temperature to allow coagulation, and centrifuged at 1300 g for 10 minutes. The serum

supernatant was transferred to new tubes, centrifuged at 16,000 g for 15 minutes to remove any residual cells and debris, and stored at −80°C (115).

Cell culture

HeLa cells were grown in RPMI basal medium with L-glutamine and supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were grown at 37°C at 5% CO_2.

HNSCC serum treatments

HeLa cells were plated in 24-well plates (Corning) at low density and allowed to attach for several hours. Medium was then removed and replaced with freshly prepared medium containing 10% human serum and no FBS. A total of 11 serum samples were used, 7 from HNSCC patients and 4 from healthy individuals. Cells were allowed to grow for 72 hours before extracting RNA.

miRNA Extraction

Total RNA was extracted from a confluent well of a 24-well plate of serum-treated cells (n=11) using RNeasy Universal Plus Mini Kit (Qiagen) according to the manual with option to include miRNA. MiRNA was extracted from 200 µL serum using miRNeasy Serum/Plasma kit (Qiagen) according to manual. RNA concentration and purity was determined using a Gen 5 plate reader (EpochTM Mircoplate Spectrophotometer, BioTek, Winooski, VT, USA).

miRNA library preparation and sequencing

Libraries for miRNA sequencing were prepared from RNA from serum and serum treated cells using NEBNEXT Small RNA Library Prep Set for Illumina (New England Biolabs). Libraries were prepared according to the manufacturer's protocol using a 6% Polyacrylamide gel for size selection. Each sample was given a unique index primer and then all serum samples and all cell samples were pooled. The quantity and quality of miRNA libraries was determined using BioAnalyzer and RNA Nano Lab Chip Kit (Agilent Technologies, Santa Clara, CA, USA), and the samples were combined in a single microcentrifuge tube and submitted to sequencing on a HiSeq 2500 instrument (Illumina Inc.).

Sequencing data was analyzed using sRNAtoolbox (148) for alignment and quantification of miRNA libraries and using EdgeR (149) for statistical analyses of differentially expressed miRNAs. MiRNAs with a FDR<0.05 and FC>1.3 were considered as up-regulated; and FDR<0.05 and FC<0.70 were considered as down-regulated. R (3.2.2) software and the Bioconductor package DESeq (1.2.0) were used for unsupervised hierarchical clustering for the 50 most expressed miRNAs (150) and miRNA gene families were identified using miRBase (151).

Target and Pathway analysis of miRNA-seq

DIANA-mirPath (v.3) was used for gene ontology (GO) analysis of biological processes and KEGG molecular pathways (152,153) using validated gene interactions of the differentially regulated miRNAs using Tarbase v7.0 database (154) considering P values lower than 0.05 as significant for pathway and GO Terms enrichment. Only KEGG pathways with at least 9 targeted genes targeted by at least 6 miRNAs were reported.

Real Time PCR

Cells were seeded in 24-well plates in normal culture medium with FBS. After cells attached, media was removed and replaced with freshly prepared media supplemented with 10% human serum in place of FBS and allowed to grow for 72 hours. Cells were lifted and RNA was extracted using RNeasy Plus Universal Mini Kit (Qiagen) according to kit manual. RNA concentration and purity was determined using a Gen 5 plate reader (EpochTM Mircoplate Spectrophotometer, BioTek, Winooski, VT, USA).

RNA was converted to cDNA using iScript cDNA synthesis kit (Bio-Rad) according to kit protocol using 40 μL reaction volumes and then samples were diluted to 80 μL. Reactions were set up in a MicroAmp® Fast Optical 96-Well Reaction Plate (Applied Biosystems) with 2 μL of cDNA, 0.2 μL each of forward and reverse primer, 12.6 μL of nuclease free water, and 5 μL of Fast SYBR Green Master Mix (Applied Biosystems) per well. Quantitative PCR was performed using a 7900 HT Fast system (Applied Biosystems) for polymerase activation at 95°C for 20 seconds followed by 45 cycles of 1 second denaturation at 95°C and 20 second extension at 62°C. Primer sequences are listed in Table 6. List of primers used to determine relative gene expression.

Beta-2-microglobulin (B2M) was used as a housekeeping gene to normalize qPCR data. Relative gene expression was calculated using the following equation: $2^{A-B}/2^{C-D}$ (A=Ct value of the gene of interest in the first control (healthy human) sample, B=Ct value of the gene of interest in each sample, C=Ct value of B2M in the first control (healthy human) sample, D=Ct value of B2M in each sample. This gives the first control (healthy human) sample a relative expression of 1 and all other samples are calculated in relation to this sample. The results of healthy individual group were

averaged and results of other samples were divided by this average to give the fold change in expression of genes of interest in cells treated with cancer serum compared with cells treated with healthy human serum (155).

Statistical analysis of relative gene expression

Relative expression of genes of interest for cells treated with healthy or cancer serum were subjected to a two-tailed t test with a 95% confidence interval and graphed with mean ± standard error of the mean (SEM) using Prism 5 software (GraphPad).

Results

HNSCC patient serums alter the miRNA profile of treated cells

In order to investigate the effects of cancer-associated circulating factors on cells in culture, we treated HeLa cells with serum from HNSCC patients and from healthy humans. Next-Generation sequencing of miRNA reveals that serum from HNSCC patients induces a different miRNA expression profile than the serum form healthy individuals. We detected a total of 377 miRNA expressed in HeLa cells and found a total of 16 miRNAs that were differentially expressed (Table 3): 12 were down-regulated and 4 were up-regulated in cells that were treated with cancer serum compared to cells treated with healthy human serum.

Key pathways are targeted by differentially expressed miRNAs

Tarbase did not contain interaction data for four of the differentially expressed miRNA that were identified, but analysis of the gene ontologies (GO) (Table 4) and pathways (Table 5) targets of the other 12 miRNA reveal that these miRNA target genes involved in key biological processes. In addition to several targeted cancer pathways, other cancer-related processes such as focal adhesion, cell cycle and apoptosis, and critical signaling pathways were identified. These data expose the potential ability of HNSCC patient serum to affect expression of genes in key cellular pathways by altering levels of regulating miRNAs.

Other critical genes are affected by exposure to HNSCC serum

In order to relate the differentially expressed miRNA to gene expression, we performed quantitative PCR to measure mRNA levels of some critical genes involved in cancer. A total of 14 genes were tested and 5 were found to have significantly reduced expression ($p<0.05$) in cells that were treated with cancer serum compared with those treated with serum from healthy individuals (Figure 32). This further demonstrates the ability of HNSCC serum to alter the expression of cancer-related genes when introduced to cells.

Discussion

Differentially expressed miRNAs are involved in cancer pathways

The miRNAs identified in this study interact with proteins that are known to be involved in oncogenic processes including proliferation, survival, and angiogenesis (Figure 33), and many have been shown to be dysregulated in cancer. Dysregulation of proteins involved in the regulation of apoptosis can have oncogenic consequences as evidenced by the many cell cycle regulating proteins that are either tumor suppressors or oncogenes. One of these proteins that is frequently mutated or dysregulated in cancer is p53, which acts as a guardian of the DNA damage cell cycle checkpoint and is responsible for initiating apoptosis when damage cannot be repaired. P53 is a vital tumor suppressor demonstrated to play a major role in head and neck cancer as well as in other types of cancer (156). In this study, we identified MDM2 as a target of miR-32-5p (Figure 33), which we found to be downregulated in cells treated with cells treated with HNSCC serum compared with those treated with healthy serum. This interaction is supported by studies demonstrating the ability of miR-32 to cause accumulation of the tumor suppressor p53 by facilitating degradation of MDM2 (157). Further, Sirt1, a target of miR-128-3p and miR-32-5p, also deacetylates p53 thereby inhibiting its transcriptional activity (162), so reduced expression of these miRNA could facilitate p53 inhibition by increasing Sirt1 expression. Notably, we also found that exposure to HNSCC serum resulted in decreased p53 expression.

Two other miRNAs found in this study to be downregulated by HNSCC patient serum exposure, miR-212-5p and miR-132-5p, also target proteins involved in cell cycle regulation. MiR-212-5p targets CCND1 and miR-132-5p targets Bcl2 (Figure 33). The protein product of CCND1, cyclin D1, cooperates with other proteins facilitate cell cycle progression from G1 to S phase while

Bcl2 inhibits apoptosis by blocking the activity of pro-apoptotic proteins like Bax, Bak, and p53. MiR-132 and miR-212 are formed by differential processing of the same pri-miRNA, and upregulation of this gene cluster was shown to increase apoptosis as well as downregulate cyclin D1 and induce cell cycle arrest (158).

Mir-128 has been shown to be downregulated in many types of cancer and has been shown to act as a tumor suppressor in HNSCC specifically. Overexpression of miR-128 in HNSCC cell lines inhibited cell growth and downregulated anti-apoptotic proteins including MDM2, Bcl2, and NFkB (159). MCL1, another anti-apoptotic member of the BCL2 family, is a target of miR-32-5p, and overexpression of miR-32 was shown to induce apoptosis (160). MiR-135-5p, one of the miRNAs found to be upregulated in cells treated with HNSCC, targets the tumor suppressor APC and has been shown to promote cell growth in colorectal cancer (161).

We found that SLC2A1, a target of miR-30c-2-3p, was downregulated in cells treated with cancer serum but not with normal serum. Although the other downregulated genes identified in this study were not identified as direct targets of the differentially expressed miRNA, they could be indirectly affected by other genes that are targeted. Several of the miRNAs identified target genes that are involved in regulating gene expression such as transcription factors and histone modifiers, potentially enabling them to pleiotropically alter gene expression in the treated cells.

HNSCC serum promotes a tumorigenic expression profile in exposed cells

In summary, 16 miRNAs were found to be differentially expressed in cells treated with HNSCC patient serum compared with cells treated with healthy human serum. These miRNAs are involved in essential cellular processes that dysregulated in cancer cells. Further, the serum altered

the expression of several cancer-related genes. While other studies have demonstrated differences in miRNA expression between healthy and cancer tissue in HNSCC as well as in serum from HNSCC patients compared with serum from healthy individuals, this study goes further to demonstrate the ability of cancer serum to alter the expression of genes and miRNA in exposed cells. This concept could have significant impact on the study of metastatic cancer as it reveals the ability of cancer-associated factors in circulation to affect the expression of genes and regulatory elements in distal cells in favor of tumorigenesis. Further, this could contribute to the systemic effects that cancer has on patient health.

Table 3. List of miRNA differentially expressed between cells treated with healthy HNSCC patient serum and with normal human serum.

miRNA	HEALTHY[1,2]	CANCER[1,3]	FC[4]	P-VALUE	FDR[5]
Down-regulated					
hsa-miR-216b-5p[6]	1945.6 ± 753.8	821.9 ± 204.0	0.42	0.0000	0.0000
hsa-miR-128-3p[6]	6633.9 ± 762.8	4384.1 ± 636.8	0.66	0.0001	0.0049
hsa-miR-216a-3p	317.0 ± 130.3	165.1 ± 20.5	0.52	0.0001	0.0049
hsa-miR-4443	50.9 ± 25.6	16.6 ± 12.1	0.33	0.0003	0.0206
hsa-miR-24-1-5p	38.7 ± 8.6	20.7 ± 4.9	0.53	0.0005	0.0223
hsa-miR-212-5p	225.6 ± 44.9	142.5 ± 18.4	0.63	0.0005	0.0225
hsa-miR-424-3p	169.9 ± 51.3	104.1 ± 13.0	0.61	0.0010	0.0330
hsa-miR-4483[6]	38.9 ± 13.1	19.5 ± 6.0	0.50	0.0009	0.0330
hsa-miR-132-5p	557.4 ± 61.2	312.6 ± 144.3	0.56	0.0012	0.0353
hsa-miR-216a-5p[6]	169.8 ± 69.6	88.1 ± 34.3	0.52	0.0016	0.0369
hsa-miR-32-5p	607.1 ± 71.7	392.8 ± 111.7	0.65	0.0015	0.0369
hsa-miR-5100	400.9 ± 74.8	264.2 ± 53.6	0.66	0.0021	0.0465
Up-regulated					
hsa-miR-31-3p	110.6 ± 13.8	246.9 ± 92.1	2.23	0.0000	0.0018
hsa-miR-143-5p	1.1 ± 1.1	7.9 ± 3.0	5.81	0.0000	0.0025
hsa-miR-30c-2-3p	709.6 ± 51.0	981.4 ± 64.2	1.38	0.0011	0.0348
hsa-miR-135b-5p	4.5 ± 2.6	13.5 ± 4.2	3.03	0.0005	0.0223

[1]miRNA reads per million (rpm)
[2]Cells treated with healthy human serum, n=4
[3]Cells treated with HNSCC patient serum, n=7
[4]Fold change in cancer serum compared with normal human serum
[5]False Discovery Rate
[6]Several differentially expressed miRNA were not found in Tarbase, so they were excluded from pathway analysis

Table 4. Gene Ontology terms for biological processes of target genes of 16 miRNAs differentially expressed in cells treated with serum from healthy individuals compared with HNSCC patients

GO CATEGORY-BIOLOGICAL PROCESS	P-VALUE	#GENES[1]	#MIRNAS[2]
Response to stress	0	330	6
Catabolic process	0	305	7
Viral process	0	124	7
Symbiosis, encompassing mutualism through parasitism	0	138	7
Biological_process	0	2006	8
Biosynthetic process	0	665	8
Cellular nitrogen compound metabolic process	0	813	8
Cellular protein modification process	0	425	9
Gene expression	0	171	9
Mitotic cell cycle	3.33E-16	93	8
Neurotrophin trk receptor signaling pathway	2.67E-13	54	5
Cellular protein metabolic process	3.89E-12	80	5
Cellular component assembly	1.44E-11	184	5
MRNA metabolic process	6.96E-11	48	5
Small molecule metabolic process	2.99E-10	307	6
RNA metabolic process	8.23E-10	53	5
Membrane organization	4.12E-09	98	5
Fc-epsilon receptor signaling pathway	2.08E-08	35	5
Nucleobase-containing compound catabolic process	3.67E-08	128	5
Macromolecular complex assembly	2.67E-07	122	5
DNA metabolic process	3.81E-06	87	2
Epidermal growth factor receptor signaling pathway	5.96E-06	38	3
Transcription, dna-templated	1.18E-05	286	3
Cell death	2.68E-05	128	4
Fibroblast growth factor receptor signaling pathway	5.45E-05	35	3
Activation of signaling protein activity involved in unfolded protein response	3.01E-04	17	4
Blood coagulation	8.64E-04	58	3
Viral life cycle	5.31E-03	14	2
Immune system process	7.60E-03	136	3
Protein complex assembly	8.01E-03	82	3
Endoplasmic reticulum unfolded protein response	8.49E-03	18	2
Cellular lipid metabolic process	1.69E-02	24	2
Termination of rna polymerase ii transcription	2.33E-02	10	2
TRIF-dependent toll-like receptor signaling pathway	2.91E-02	18	3

[1]Number of genes in each process targeted by miRNAs
[2]Number of miRNAs targeting genes in each process

Table 5. KEGG pathways of target genes of 16 miRNAs differentially expressed in cells treated with serum from healthy individuals compared with HNSCC patients

KEGG PATHWAY	P-VALUE	#GENES[1]	#MIRNAS[2]
Pathways in cancer	1.23E-02	95	11
PI3K-AKT signaling pathway	3.38E-02	82	12
HTLV-I infection	3.24E-02	68	12
Focal adhesion	2.35E-03	62	11
Proteoglycans in cancer	1.51E-06	60	12
Epstein-Barr virus infection	9.00E-03	58	12
Viral carcinogenesis	1.80E-03	56	11
Endocytosis	2.51E-02	52	11
FOXO signaling pathway	2.35E-03	46	11
Hepatitis B	3.62E-03	46	12
Protein processing in endoplasmic reticulum	2.51E-02	45	10
RNA transport	4.32E-02	44	11
Ubiquitin mediated proteolysis	2.48E-02	41	10
Cell cycle	2.35E-03	40	10
Transcriptional misregulation in cancer	4.32E-02	40	10
Signaling pathways regulating pluripotency of stem cells	3.43E-02	39	11
Thyroid hormone signaling pathway	8.07E-03	37	11
Neurotrophin signaling pathway	1.38E-02	36	11
AMPK signaling pathway	4.32E-02	36	11
Oocyte meiosis	1.69E-04	35	10
Small cell lung cancer	4.01E-04	33	10
Choline metabolism in cancer	5.34E-03	33	10
Prostate cancer	2.35E-03	32	11
Adherens junction	7.74E-07	30	10
Estrogen signaling pathway	3.62E-03	30	11
Bacterial invasion of epithelial cells	9.64E-05	29	10
Erbb signaling pathway	1.62E-02	29	9
Chronic myeloid leukemia	4.96E-04	28	10
Progesterone-mediated oocyte maturation	3.28E-02	28	12
Salmonella infection	4.05E-02	27	11
Glioma	3.37E-06	26	9
Colorectal cancer	6.49E-05	26	11
Apoptosis	7.44E-03	24	9
P53 signaling pathway	9.00E-03	24	10
Prolactin signaling pathway	9.00E-03	24	10
Non-small cell lung cancer	1.69E-04	23	10

KEGG PATHWAY	P-VALUE	#GENES[1]	#MIRNAS[2]
Shigellosis	2.35E-03	23	10
Pancreatic cancer	3.42E-03	23	9
Renal cell carcinoma	3.50E-02	22	8
Epithelial cell signaling in helicobacter pylori infection	4.62E-02	22	7
Melanoma	2.96E-02	21	8
Endometrial cancer	2.35E-03	20	11
Central carbon metabolism in cancer	3.24E-02	20	8
Synaptic veside cyde	3.38E-02	18	7
Bladder cancer	1.61E-02	15	7
Lysine degradation	3.38E-02	12	6
Thyroid cancer	5.23E-03	11	7
Fatty acid metabolism	5.16E-03	9	9

[1]Number of genes in each pathway targeted by miRNAs
[2]Number of miRNAs targeting genes in each pathway

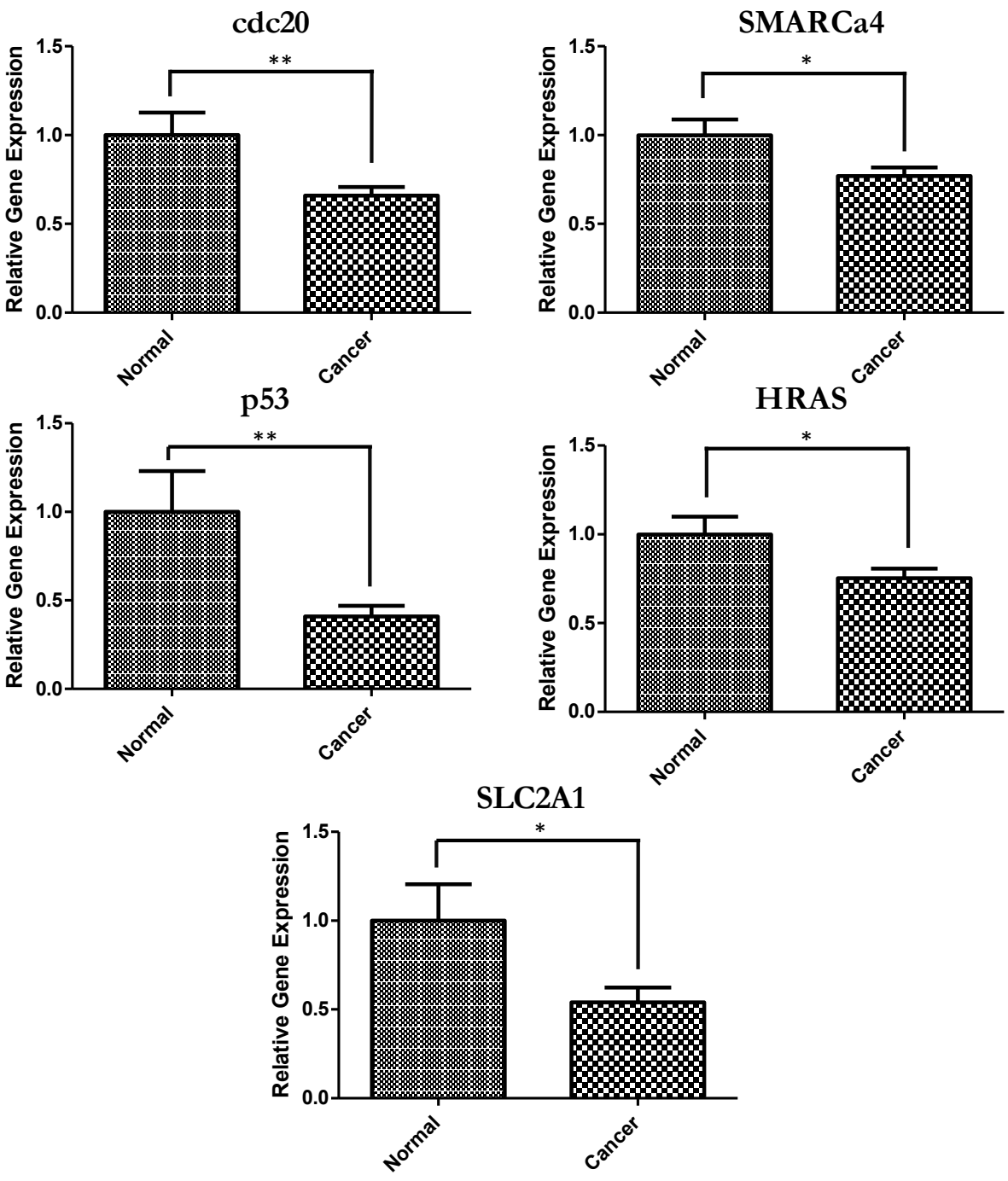


Figure 32. Gene expression changes in cells exposed to HNSCC patient serum.

Quantitative PCR was used to determine relative gene expression of several cancer-related genes. Primers used for each analysis are listen in Table 6. SLC2a1 (p=0.0198), p53 (p=0.0011), cdc20 (p=0.0043), HRAS (p=0.0306), Smarca4 (p=0.0235).

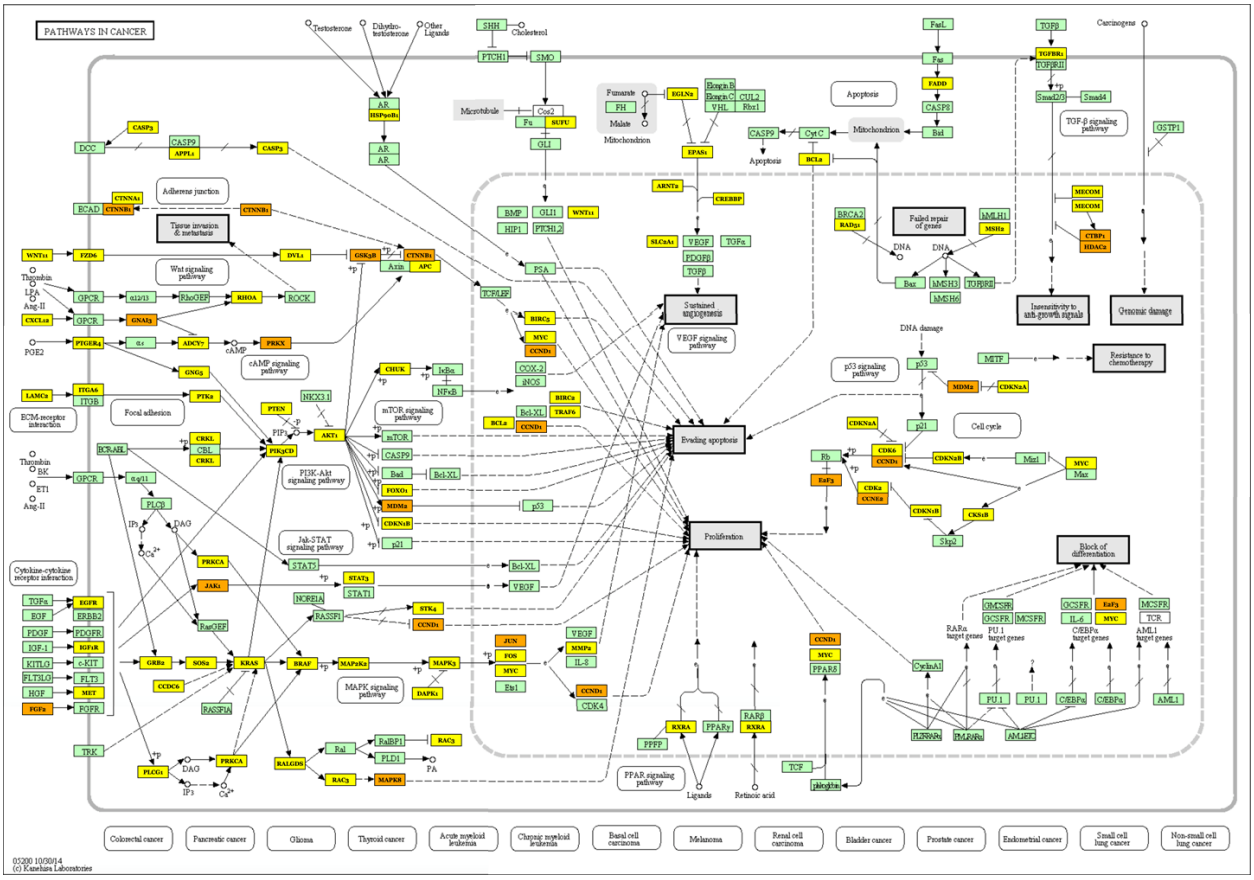


Figure 33. KEGG pathway (152) analysis: Pathways in cancer.

Diagram of pathways in cancer genes and interactions. Genes highlighted in yellow are targeted by one of the differently expressed miRNA and genes highlighted in orange are targeted by more than one miRNA.

Table 6. List of primers used to determine relative gene expression.

GENE	PRIMER	SEQUENCE
B2M	F'	5'-GAGTATGCCTGCCGTGTGAA-3'
B2M	R'	5'-CGGCATCTTCAAACCTCCAT-3'
TP53	F'	5'-GTGCAGCTGTGGGTTGATTC-3'
TP53	R'	5'-GCCAGACCATCGCTATCTGA-3'
RB1	F'	5'-TCAGAAGGTCTGCCAACACC-3'
RB1	R'	5'-CAGAAGTCCCGAATGATTCACC-3'
CDC20	F'	5'-AATGCGCCAGAGGGTTATCA-3'
CDC20	R'	5'-CGGCCAGTACATTCCCAGAA-3'
SLC2A1	F'	5'-GAACTCTTCAGCCAGGGTCC-3'
SLC2A1	R'	5'-ACCACACAGTTGCTCCACAT-3'
DNMT1	F'	5'-GATCGAGACCACGGTTCCTC-3'
DNMT1	R'	5'-CGGCCTCGTCATAACTCTCC-3'
DNMT3A	F'	5'-GGGGGAGGCACTTGACAC-3'
DNMT3A	R'	5'-CTCTGTCAGCCTGTGGGTG-3'
DNMT3B	F'	5'-ATAAGTCGAAGGTGCGTCGT-3'
DNMT3B	R'	5'-TGTGCGTCTTCGAGTCTTGT-3'
CDKN2A	F'	5'-TGCCCAACGCACCGAAT-3'
CDKN2A	R'	5'-CGGGTGAGAGTGGCGG-3'
SMARCA4	F'	5'-CGCAAGGAGGTGGACTACAG-3'
SMARCA4	R'	5'-AGCGTGCCCTCCTCGAT-3'
CCND1	F'	5'-GCCGAGAAGCTGTGCATC-3'
CCND1	R'	5'-GGCCAGGTTCCACTTGAG-3'
GFP	F'	5'-GCTCGATGCGGTTCACCAG-3'
GFP	R'	5'- GCTCGATGCGGTTCACCAG-3'
HRAS	F'	5'-GGACGAATACGACCCCACTAT-3'
HRAS	R'	5'-TGTCCAACAGGCACGTCTC-3'
NOTCH1	F'	5'-AGCCTCAACGGGTACAAGTG-3'
NOTHC1	R'	5'-GCCACTGGTCATGTCTTTGC-3'
MDM2	F'	5'-AGGAGATTTGTTTGGCGTGC-3'
MDM2	R'	5'-TGAGTCCGATGATTCCTGCTG-3'
PTEN	F'	5'-ACTTGCAATCCTCAGTTTGTGG-3'
PTEN	R'	5'-AACTTGTCTTCCCGTCGTGT-3'

CHAPTER FIVE: CONCLUSIONS

Summary

The studies outlined here provide evidence for tumorigenic processes involving epigenetic regulation. We have demonstrated DNA methylation revision following DNA repair and epigenetic activation of gene expression and aberrant miRNA and gene expression following exposure cancer serum. The identity of the hypomethylating protein in HNSCC patient serum remains to be determined.

Conclusions

Interconnections between gene regulation processes

The processes of DNA methylation, chromatin remodeling, and miRNA expression are interdependent processes that concurrently regulate gene expression and are involved in tumorigenesis. DNA methylation causes chromatin condensation through interaction with histone modifiers (14-16), and chromatin structure can affect DNA methylation by blocking the accessibility of demethylating enzymes (121,126-128). In addition to regulating gene expression, these processes have been shown to regulate the expression of miRNAs (135-137) and, conversely, proteins involved in this process can be regulated by miRNAs. These epigenetic and miRNA regulation mechanisms form a network of processes with interconnected regulatory loops. Dysregulation of any of these processes may also affect the others and together can contribute to tumorigenesis. The

methylation revision following repair by NHEJ and the cancer-associated circulating protein that were described in this study demonstrate the connection between these regulatory processes and the development of cancer.

Identifying new targets for cancer epi-therapeutics

One important property of epigenetic alterations that makes them an ideal therapeutic target is that they are reversible. This is in contrast to genetic mutations that are embedded in the DNA sequence. In fact, hypomethylating drugs including 5'Aza-2'-deoxycitidine (Decitabine), and 1-β-D-ribofuranosyl-2(1H)-pyrimidinone (Zebularine), have been shown to have positive effects when used to treated cancer cells. The FDA has approved the use of Decitabine for treating myelodysplastic syndrome and Zebularine for treating hematological malignancies (24,27,33,67). Studies have also shown that antisense and small interfering RNA (siRNA) targeting DNA methyltransferase mRNA can inhibit growth of colon and renal cell carcinoma cells (27,33). Further, HDAC inhibitors, have been shown to selectively target and promote death in tumor cells. This lead to approval of Voronistat, an HDAC inhibitor approved to treat cutaneous T-cell lymphoma. In addition to increasing our knowledge of carcinogenic processes, uncovering the details of the interaction between epigenetic and gene regulation processes observed in these studies can potentially reveal new diagnostic and therapeutic targets in the inception, progression, and metastasis of cancer.

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