WIF1 IS A FREQUENT TARGET FOR ABERRANT HYPERMETHYLATION IN CERVICAL SQUAMOUS CELL CARCINOMA

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2010

ACKNOWLEDGMENTS

I am most thankful to both of my mentors, Mike and Kevin, for believing in me and constantly stimulating my brain. Mike is an extremely intelligent individual who has continuously inspired me to think outside of the box, while Kevin is one of the most dedicated and hardworking individuals I know. Together you make the perfect boss.

To my committee members: Jim Resnick and Paul Gulig, thank you for your guidance and support.

My deepest appreciation to my lab family: Carolina Pardo, Russell Darst, Nancy Nabilsi, Santhi Pondugula, and Chris Hoffman. Thank you for putting up with my endless questions and constant interruptions. I also wish to thank my extended lab family: Lisa Dyer for the mass of cell culture work and Ryan Skehan for his wealth of computer knowledge.

I want to acknowledge the Moffitt Cancer Center and Erin Siegel in Tampa, Florida and wish to thank Bridget Riggs for her hard work and dedication.

I am thankful to Joyce Conners and all of her hard work in assisting every Master's student along the road from acceptance to graduation.

I thank my Dad for trying to understand what exactly I'm researching and to my Mom for not trying to have me explain what I do. I wish to express thanks to John Barli for allowing me the opportunity to support myself through school, and to everyone else who has come in and out of my life to get me where I am today.

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LIST OF ABBREVIATIONS

APC	adenomatous polyposis coli
ATP	adenosine-5'-triphosphate
CCD	charged couple device
CK1	casein kinase 1
CLB	cell lysis buffer
CIN	cervical intraepithelial neoplasia
CIS	carcinoma *in situ*
CpG	cytosine guanine dinucleotide
CRB	cell resuspension buffer
DAB	diaminobenzidine
dg.dH20	degassed distilled water
dH20	distilled water
DKK	Dickkopf
DNA	deoxyribonucleic acid
DNMT	deoxyribonucleic acid methyltransferase
dNTP	deoxyribonucleotide triphosphate
Dvl	Dishevelled
EGF	epidermal growth factor
FBS	fetal bovine serum
FFPE	formalin-fixed paraffin-embedded
Fz or Fzd	Frizzled receptor
GSK3β	glycogen synthase kinase 3β
GTPase	guanine triphosphotase
HAT	histone acetyltransferase

HDAC	histone deacetylase
HDM	histone demethylase
HMGA2	high mobility group AT-hook 2
HMT	histone methyltransferase
HPV	human papillioma virus
hr	hour(s)
IHC	immunohistochemistry
JNK	c-jun N-terminal kinases
LEF	lymphoid enhancer factor
LRP	lipoprotein receptor-related protein
MB	methylation buffer
MAPit	methyltransferase accessibility protocol for individual templates
MAZ	MYC-associated zinc finger
min	minute(s)
NF-kB	nuclear factor-kappa B
NFR	nucleosome-free region
NuRD	nucleosome remodeling and deacetylase
Pap	Papanicolau
PCR	polymerase chain reaction
PPi	pyrophosphate
Rb	retinoblastoma protein
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SCC	squamous cell carcinoma
sec	second(s)

sFRP	secreted Frizzled receptor protein
SWI/SNF	switch/sucrose non-fermentable
TCF	T cell factor
TSG	tumor suppressor gene
TSS	transcription start site
UICC	International Union Against Cancer
WD	WIF1 domain
WIF1	Wnt inhibitor factor 1
WHO	World Health Organization

Abstract of Thesis Presented to the Graduate School
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August 2010

Chair: Michael Kladde
Major: Medical Sciences

Aberrant activation of the Wnt/β-catenin signaling pathway is a prominent oncogenic mechanism in numerous cancer types, and evidence indicates this pathway is of importance in human cervical cancer. Wnt Inhibitory Factor 1 (WIF1) is one of several secreted antagonists that bind to Wnt. Although it has been described as a tumor suppressor in various types of cancer, regulation of the WIF1 gene has not been examined in human cervical cancer. Here we show that *WIF1* was unmethylated and expressed in normal cervical epithelium and aberrantly methylated in high-grade, squamous cell carcinomas. Three of four cervical tumor cell lines showed low or no *WIF1* expression. In all three, expression increased when cells were cultured with a DNA demethylating drug, indicating epigenetic silencing of *WIF1*. Interestingly, differences were seen in chromatin structure between expressing and non-expressing cell lines as shown by the chromatin footprinting technique, MAPit. The *WIF1* promoter was aberrantly methylated in ten of seventeen high-grade squamous cell cervical tumors, compared to paired normal tissue. Immunohistochemistry of normal stratified squamous cervical epithelium revealed that *WIF1* expression was high in peribasal cells, but gradually diminished toward the superficial epithelial layer. WIF1 protein was not detectable in tumors with high *WIF1* methylation and was absent in two of the seven unmethylated tumors. Thus, although *WIF1* was hypermethylated in

most cervical cancer samples, other mechanisms may also contribute to its repression. Our findings establish the *WIF1* gene as commonly hypermethylated and a possible target for epigenetic silencing in squamous cell carcinoma.

CHAPTER 1
BACKGROUND

Cervical Cancer

In 2009, the American Cancer Society estimated within a year approximately 4,070 women would die from cervical cancer in the United States. About 11,270 new cases of invasive cervical cancer would be diagnosed, and four times as many women would have a non-invasive cervical cancer [1]. Although cervical cancers can develop in the mucus-producing gland cells of the endocervix, most form in the lining of the ectocervix, which is composed of stratified squamous epithelium. The area defining the border between the endocervix and ectocervix is most susceptible to dysplasia, and is termed the transformation zone (Figure 1-1A). Dysplasia is characterized by four major pathological microscopic changes: unequally sized cells, abnormally-shaped cells, an unusual number of cells that are currently dividing and hyperchromatism (degeneration of cell nuclei or increased staining capacity) [2]. If molecular changes occur in the basal cells where these cells originate from, they can promote the normal tissue through increasing grades of dysplasia and if not caught, cervical carcinoma. The naming and classification of cervical carcinoma precursor lesions has changed several times. Originally, the World Health Organization (WHO) classified lesions as mild, moderate, and severe dysplasia or carcinoma *in situ* (CIS). These precursor lesions were graded depending on the degree of disruption of epithelial differentiation. These classifications were later standardized as cervical intraepithelial neoplasia (CIN), with mild dysplasia designated CIN1 and severe as CIN3. The most recent classification is the Bethesda System, which divides all cervical epithelial precursor lesions into two groups [3]: low-grade squamous intraepithelial lesions that correspond to CIN1 (Figure 1-1B) and high-grade squamous intraepithelial lesions that include CIN2 and 3 (Figure 1-1C). These cellular changes can be detected by the Papanicolau test (Pap test) and treated to

prevent the development of cancer. Treatment consists of surgery in early stages or chemotherapy and radiotherapy in advanced stages of the disease.

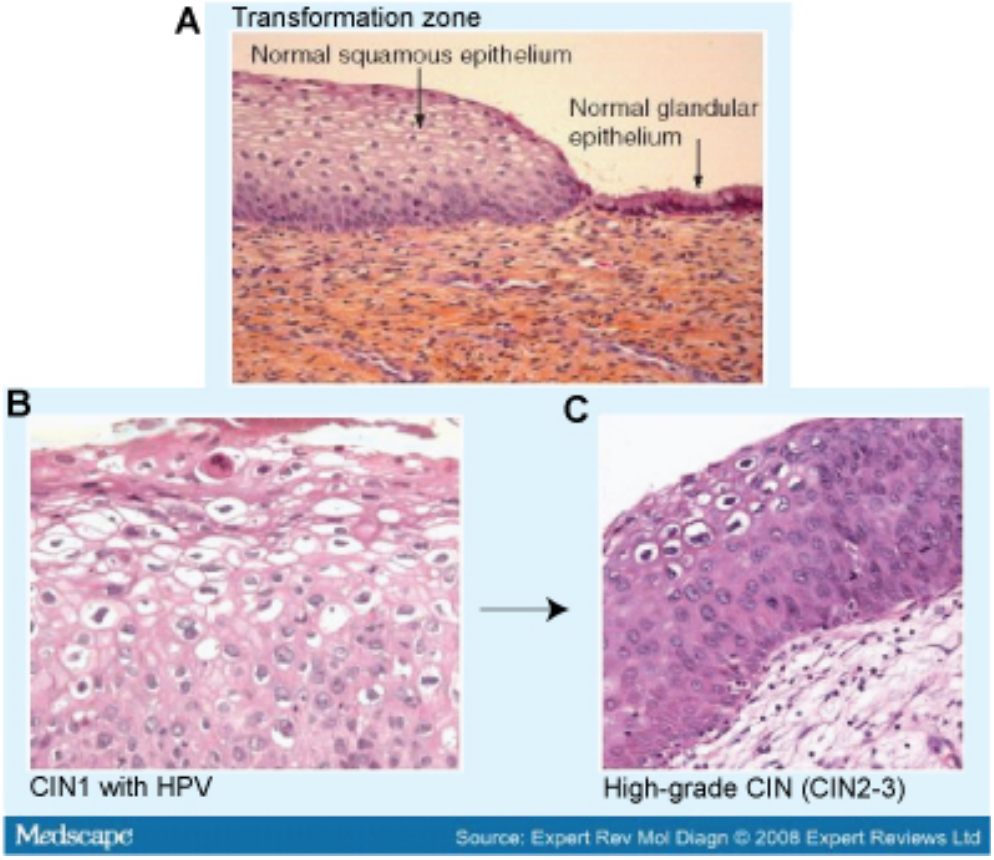

Figure 1-1. Normal Cervical Epithelium, CIN1 and CIN2-3[1] (A) Staining of the transformation zone showing both squamous and glandular epithelium on the surface and the underlying parenchyma. Nuclei are stained dark purple. (B) Squamous cells that have progressed to CIN1: moderate nuclear enlargement and rounded cells. (C) CIN 2-3 cells have large nuclei occupying the majority of the cell and increased staining.

Human Papillomavirus

Infection with oncogenic human papillomavirus (HPV) has been shown to be the primary etiological factor for cervical cancer and its precursor lesions [5]. Papillomavirus is a small deoxyribonucleic acid (DNA) virus that induces a variety of proliferative lesions. HPV infections

[1] adapted with permission from Ref. 4. Lie, A.K. and G. Kristensen, *Human papillomavirus E6/E7 mRNA testing as a predictive marker for cervical carcinoma.* Expert Rev Mol Diagn, 2008. **8**(4): p. 405-415.

are often transient, but persistent infections, especially with oncogenic HPV or high-risk types, can further increase the likelihood of cervical dysplasia [6]. HPV-induced oncogenesis in cervical carcinoma derives from the properties of the expressed viral gene products. Integration of the viral circular DNA into the patient's genome often disrupts the viral E2 gene, which leads to deregulation and expression of the E6 and E7 viral proteins [7].

The E6 gene product is able to interact with many cellular proteins, but it primarily binds with the human tumor suppressor p53 protein. E6 forms a complex with the transcription factor AP1, binds to p53, and induces its degradation through the ubiquitin protein ligase pathway. The p53 protein has many names, including "the guardian of the genome", due to its role in maintenance of genome stability by preventing the transmission of DNA mutations that arising due to cellular stress. Cells with dysfunctional p53 are unable to respond normally to DNA damage and thus allow accumulation of genomic mutations [8, 9].

Viral E7 disrupts normal cell cycle regulation through its interaction with the tumor suppressor retinoblastoma (Rb) protein. Under normal cell cycle regulation, Rb forms complexes with the E2F-1 family of transcription factors thereby inhibiting their function. Viral E7 binds to Rb thus disrupting the inhibitory complex resulting in the release of active E2F-1. Subsequently E2F-1 binds and initiates transcription of target genes that facilitate the G1/S transition and begin S phase. The E7 proteins of the high risk HPVs, such as HPV-16 and HPV-18, bind Rb with 10-fold higher affinity than do the E7 proteins of the low risk HPV types. The difference in binding affinity correlates with the transforming potential of the different E7 proteins. HPV infection alone is not sufficient to induce malignant transformation. Other significant cofactors contribute to the multi-step process of tumor formation, such as individual genetic variations as well as environmental factors; however, such cofactors are not important in the absence of HPV

[10]. The combined dysregulation of p53 and Rb allows cells with DNA damage to proceed into S phase facilitating the accumulation of genetic mutations, which is the basis for carcinogenesis.

Human Cell Culture

Much of what is known about the correlation between cervical cancer and HPV infection comes from cell culture studies [11]. The practice of tissue culture is commonly used to study all types of cancer, including those of the cervix. Cell culture is a useful tool for the study of *in vitro* animal cell biology because it allows access to a highly-controlled, easily-manipulated environment. There are a number of cultured human cervical cell lines that are commonly used to study cervical cancer. The cell lines used in this study are C-33A, CaSki, HeLa 229 and SiHa. The C-33A cell line is one of a series of lines derived by N. Auersperg from cervical cancer biopsies [12]. The line, when originally cultured, exhibited an epithelial morphology, and karyological instability that was observed with continued passage. The line is pseudodiploid with a modal chromosome number of forty-six, occurring in 70% of cells examined. The cells are negative for human papillomavirus DNA and RNA. The CaSki line was established with cells from a metastasis in the small bowel mesentery of a woman with epidermoid carcinoma of the cervix. These cells are reported to contain integrated HPV-16 as well as sequences related to HPV-18. HeLa 229 cells were created from an epithelial cervical adenocarcinoma that contains HPV-18. They have a modal chromosome number of 82. HeLa 229 cells are essentially the same as the original HeLa cell line; however, they are relatively resistant to infection by polioviruses. The SiHa cell line was established from fragments of a primary tissue sample obtained after surgery from a Japanese patient with grade-II squamous cell carcinoma that contains one to two copies of HPV-16 per cell [12]. The HPV-positive cervical cancer cell lines express normal Rb and low levels of wild-type p53 proteins, which are presumed to be altered in function as a consequence of association with HPV E7 and E6 oncoproteins, respectively. In C-33A, the Rb

protein is present but abnormal in size. These cells also overexpress a p53 DNA binding mutant with an arginine to cysteine substitution in the DNA binding domain [13]. Characterization of these cell lines further supports that inactivation of the normal functions of the tumor suppressor proteins Rb and p53 are important steps in human cervical carcinogenesis, either by genetic mutation or through inactivation by the HPV E6 and E7 oncoproteins.

Epigenetics

Both genetic and epigenetic changes are components of tumorigenesis [14]. Epigenetics is the study of the heritable changes in gene expression that occur independent of the DNA coding sequence. Epigenetic mechanisms that modify chromatin structure can be divided into four main categories: DNA methylation, covalent histone modifications, non-covalent mechanisms such as nucleosome remodeling, and small, non-coding ribonucleic acids (RNAs) [15]. Collectively, these modifications regulate chromatin accessibility and its compactness. Their interplay controls how the genome manifests itself in different cell types and developmental stages. Inappropriate alterations of components of these mechanisms are commonly found in diverse disease states, including cancer.

DNA Methylation

In mammals, DNA methylation primarily occurs at the five position of the cytosine ring when directly followed by guanine (CpG). The majority of the genome is CpG poor; however, there are variable stretches of DNA that contain a high frequency of CpG dinucleotides within the genome and are thus designated CpG islands [16]. CpG dinucleotides that are not located in CpG islands are sparsely distributed, highly methylated, and generally found in regions of large repetitive sequences such as retrotransposon elements and centromeric repeats [17]. CpG islands are more commonly found in the 5' end of the gene proximal to the transcription start site (TSS) and occupy approximately sixty percent of all human gene promoters [18]. The majority of CpG

islands are generally unmethylated and found in differentiated tissues. Promoters containing CpG islands that become hypermethylated during development result in long-term transcriptional silencing. Classic examples of such naturally occurring epigenetic silencing mechanisms are X-chromosome inactivation and gene imprinting [19, 20].

In mammals, DNA methylation is catalyzed by two families of enzymes designated DNA methyltransferases (DNMTs): DNMT1 and DNMT3. The threeknown membersof the DNMT3 family are DNMT3A, 3B and 3L. DNMT3A and DNMT3B can modify unmethylated and hemimethylated CpG base pairs at the same rate, and are thus referred to as *de novo* methyltransferases. Little is known about the regulation of *de novo* DNA methylation or which proteins are involved. It is known that DNMT3L, although catalytically inactive, interacts with DNMT3a and DNMT3b and the complexes co-localize in the nucleus [21]. DNMT3L may also participate in transcriptional repression [22]. Maintenance methyltransferases maintain the methylation patterns established by the *de novo* methyltransferase by methylating the nascent or hemimethylated DNA strand following replication. DNMT1 is known as the maintenance methyltranferase because it localizes to the replication foci and interacts with the proliferating cell nuclear antigen to methylate the newly-synthesized DNA strand [23]. DNMT1 and DNMT3s work together in a complex relationship to maintain existing and establish new cytosine methylation in mammals.

Histone Modifications

The majority of the mammalian genome is normally packaged in a transcriptionally-repressive chromatin state. This type of chromatin is heavily methylated and forms repeating units of nucleosomes. Nucleosomes are made up of an octamer of four core histone proteins with 147 base pairs of DNA wrapped around the octamer. H3, H4, H2A and H2B are the core histone proteins within the nucleosome [24]; however, several histone variants also exist. For instance, it

has been seen at active or poised genes that H2A and H3 are replaced by H2A.Z and H3.3 histone variants, respectively [25]. The histone amino-terminal tails are sites of frequent post-translational covalent modifications including acetylation, methylation, ubiquitylation, sumoylation and phosphorylation at specific residues [26]. These modifications regulate key cellular processes such as transcription, replication and repair.

Nucleosome Positioning

Histone modifications work by either changing the accessibility of chromatin or by recruiting and/or inhibiting binding by non-histone effector proteins. Families of enzymes coordinate covalent histone modifications to precise residues. Histone acetyltransferases (HATs) and histone methyltransferases (HMTs) add acetyl and methyl groups, respectively, while histone deacetylases (HDACs) and histone demethylases (HDMs) remove such marks from the histone tails. Interactions between the DNA methylation machinery and histone-modifying enzymes occur at multiple levels to affect gene expression. DNA methylation itself can affect histone methylation and acetylation through proteins such as methyl CpG binding protein 2 (MeCP2) recruiting HMTs and HDACs [27, 28]. Conversely, HMTs can direct DNA methylation to specific genomic targets by directly recruiting DNMTs to stably silence genes [29, 30], and by regulating the stability of DNMT-DNA interaction [31]. DNMTs can also recruit HDACs and methyl-binding proteins to achieve gene silencing and chromatin condensation [32, 33]. The histone modification mechanism of inheritance is still not fully understood. Whether histone marks survive replication has yet to be answered, but it is thought that chromatin states are metastable because they self perpetuate due to the network of DNMTs, HMTs, and HDACs that are recruited by the heritable DNA methylation. Regardless, it is widely accepted that the deacetylated and methylated state of the histone tails maintain nucleosomes in a tightly-compacted and transcriptionally-silent state, though exceptions have been found [34].

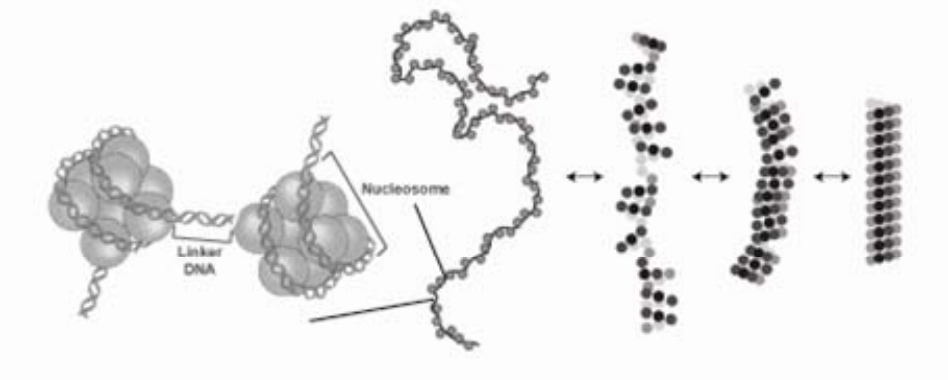

Figure 1-2. Diagram of Open and Closed Chromatin[2] Chromatin is shown in a closed state that is representative of the proposed 30 nm chromatin fiber (far right). This tight packing of nucleosomes is refractory to transcription because it impairs DNA binding by transcription factors. The cell is able to change the state of chromatin to allow access to DNA by a combination of nucleosome-modifying enzymes, DNA binding factors and nucleosome-remodeling activities. Open chromatin is represented as a beads-on-a-string configuration with a close up of two nucleosomes (far left). In open chromatin, variable lengths of linker DNA are observed between nucleosomes.

Only a small fraction of the genome, known as euchromatin, is transcriptionally active. Both covalent and non-covalent mechanisms, such as nucleosome remodeling, regulate gene expression by altering the accessibility of chromatin to transcription factors. Nucleosomes around the promoter are more widely spaced than in heterochromatin and generally contain heavily acetylated histones. It is generally thought that nucleosomal DNA resists transcriptional activation, which requires the assembly of bulky transcription machinery onto the TSS. It has long been hypothesized that nucleosomes need to be removed for active transcription [35], and there are current models depicting this in yeast [36, 37] and mammalian systems [38, 39]. In the human genome, it has been shown that nucleosomes immediately upstream of TSSs are depleted upon activation of inducible genes. The adenosine-5'-triphosphate (ATP)-dependent chromatin

[2] adapted from 44. Gilbert, N. and B. Ramsahoye, *The relationship between chromatin structure and transcriptional activity in mammalian genomes.* Brief Funct Genomic Proteomic, 2005. **4**(2): p. 129-42.

remodeling complex switch/sucrose non-fermentable (SWI/SNF) is thought to regulate promoter nucleosome eviction by sliding and/or removing nucleosomes [40, 41]. Indeed, nucleosome-free regions (NFRs) are found at the 5' and 3' ends of genes [42, 43]. Packaging of DNA into chromatin by the formation of nucleosomes is vital to gene regulation and the organization of such is only beginning to be understood.

Epigenetics in Cancer

Epigenetic alterations involve genome-wide losses and regional gains of DNA methylation as well as altered patterns of histone modifications and the enzymes that regulate them; however, the heritability of histone modifications is inconclusive since their mechanism of inheritance is currently unknown [45]. These aberrant epigenetic events have been observed in cancer, but each is currently being debated as to whether it is a foundation for or rather the consequence of cancer.

Aberrant Methylation

The least understood of these epigenetic alterations is genome-wide loss of methylated cytosine residues. Global DNA hypomethylation is most often observed within repetitive sequences dispersed throughout the genome. Recently, hypomethylation has also been observed within the body of genes [46]. Aberrant gene silencing is the more commonly studied cancer-associated epigenetic event. Aberrant gene silencing is the abnormal transcriptional silencing of genes by either direct methylation of the gene promoter or modifications to the chromatin structure. The study of hypermethylation is generally focused on tumor suppressor genes (TSG). TSGs are genes that normally inhibit cell growth. TSG proteins are found in many different cellular pathways involving cell cycle checkpoint responses, detection and repair of DNA damage, mitogenic signaling, differentiation and migration, and tumor angiogenesis [47]. In most mammalian genes, the CpG islands are normally free of DNA methylation. In cancer cells,

however, various TSG promoters have densely methylated CpG islands resulting in a loss of gene transcription [48]. This loss of transcription can act as one or possibly as both hits needed in Knudson's 'two hit hypothesis' [49]. Knudson's hypothesis that two hits are required for the full inactivation of a tumor-suppressor gene has been shown to be fundamentally correct in almost all cases of human cancer, but it is now known that the hit is not always a genetic mutation. The mechanism for aberrant methylation is largely unknown. It might be caused by dysregulation of the DNMTs or other chromatin binding proteins. Despite its unknown cause, aberrant DNA methylation is an important mechanism that is exploited by cancer.

Chromatin Changes in Cancer

Aberrant DNA methylation is one of a series of complex epigenetic silencing mechanism that lead to alterations in gene expression through changes in chromatin structure. Experiments showing that epigenetic gene silencing occurs in the absence of detectable CpG methylation indicated that mechanisms other than DNA methylation modulate gene silencing [50, 51]. It is now hypothesized that nucleosome remodeling works in concert with DNA methylation and histone modifications to drive gene silencing. There are many possible mechanisms by which this could take place in cancer. For example, the nucleosome remodeling and deacetylase (NuRD) complex facilitates recruitment of the polycomb repressive complex 2 and DNMT3A to target gene promoters leading to their permanent silencing by establishing a repressive chromatin state [52]. Components of the NuRD complex have been linked directly to oncogenesis [53]. In addition, it has been shown that alterations in the SWI/SNF complex are associated with cancer development [54]. Interactions between the DNA methylation machinery and histone-modifying enzymes further enhance the complexity of epigenetic regulation of gene expression. More is known about the maintenance of tumor suppressor gene (TSG) methylation, but we are slowly starting to understand how aberrant DNA methylation may be initiated.

Epigenetic alterations are important for the development and progression of malignant diseases. In cervical cancer, alterations in DNA methylation are thought to be associated with integration of viral DNA. The integrated viral DNA of Hepatitis B/C virus infections of the liver and Epstein-Barr virus infections in stomach cancer are known to alter the DNA methylation status of the host genome [55, 56]. HPV E7 protein has been reported to directly interact with DNMT1 and stimulate its enzymatic activity [57]. This may be the cause of hypermethylation of TSGs that has been seen in cervical carcinoma [58]. Reports that DNMT1 protein expression is increased even in low-grade cervical intra-epithelial neoplasias compared with normal squamous epithelium, and further increased in higher-grade cervical intra-epithelial neoplasias and squamous cell carcinomas of the uterine cervix, indicate that this may act as an initiating event in cervical carcinogenesis [59]. The regulation of DNA methyltransferases may be altered by viral oncogenes through interactions with Rb-associated proteins [60]. Furthermore, E6, E7, and their interactions with host proteins drive the cell cycle to ignore normal DNA regulation checkpoints, which leads to genomic instability [61]. These virally-induced alterations in host gene structure contribute to the molecular pathogenesis of HPV-associated cancers.

As the role of epigenetics in cancer becomes clearer and the interrelationships between DNA methylation and chromatin components are increasingly understood, the possibility of epigenetic approaches to cancer prevention, detection and therapy arise. A number of epigenetic alterations occur during all stages of cervical carcinogenesis. From a diagnostic point of view, because epigenetic abnormalities occur very early in the carcinogenic process they can potentially be exploited as molecular markers for early detection. Assessment of hypermethylated genes in primary tumors could potentially serve as a prognostic factor or as a means of predicting response to radiation, chemotherapy or transcriptional agents. The focus of

this project is to determine if Wnt inhibitory factor, an antagonist of the Wnt pathway, exhibits epigenetic aberrations in cervical cancer that may be exploited for future cancer therapies.

Wnt Pathway

The Wnt extracellular signaling pathway, wingless in *Drosophila*, is a highly-conserved signal transduction pathway that mediates a wide range of biological processes. From hydra to humans, Wnt signals control multiple aspects of development, including the proliferation, fate specification, polarity and migration of cells [62, 63]. There are at least two distinct pathways of Wnt signaling. The first is the Wnt/β-catenin pathway named for the molecule that activates it, Wnt. The β-catenin pathway of Wnt signaling is also commonly referred to as the 'canonical' pathway. The second is the non-canonical or β-catenin-independent pathway. Two decades of research have given us a firm understanding of the Wnt pathway. In addition, activating mutations in components of the Wnt signaling pathway have been found to play a role in oncogenesis [64, 65].

Wnt Agonists and Antagonists

There are various agonists and antagonists that act on the Wnt signaling pathway. To date, nineteen Wnt ligands have been found in mammals. They are cysteine-rich proteins of approximately 350-400 amino acids and contain an N-terminal signal peptide for secretion. Wnts are glycosylated and lipid modified in the endoplasmic reticulum, and subsequently escorted by Wntless from the Golgi to the plasma membrane for secretion. After secretion, mature Wnts either bind to heparin sulfate proteoglycans and lipoprotein particles, or form multimers with each other. Wnt antagonists can be divided into two functional classes [66]: the secreted Frizzled-related protein (sFRP) class and the Dickkopf class. Members of the sFRP class include the sFRP family, Wnt inhibitory protein (WIF1) and Cerberus all of which bind directly to Wnts, thereby altering Wnt's ability to bind to one of its receptor complexes. Only sFRPs are able to

bind both Wnts and the Wnt receptor Frizzled. The Dickkopf (DKK) class of inhibitors binds directly to the LRP5/6 co-receptor of the Wnt receptor complex (Figure 1-3B).

Wnt Receptors

The Wnt receptor complex that activates the canonical pathway contains two components: the family of seven-pass transmembrane Frizzled (Fz or Fzd) receptors and a co-receptor from the low-density lipoprotein receptor-related protein 5/6 family (LRP5 or LRP6). There are ten Fz genes in mammals which likely have functional redundancy and have varying capabilities to activate Wnt signaling when co-overexpressed with Wnt and LRP5/6 [67]. Either one of two single-span transmembrane proteins, LRP5 or 6, are also needed. In vitro studies support the model that Wnt induces the formation of Fz-LRP5/6 complex [68]; however, this has yet to be clearly shown in vivo. A particular Wnt may activate canonical and/or noncanonical pathways depending on the receptor complement [69], though it remains unclear whether the noncanonical Wnt pathway also requires LRP5/LRP6.

Canonical Pathway

The Wnt/β-catenin pathway, also referred to as the 'canonical' pathway, is activated when a Wnt ligand binds a member of the Fz receptor and the co-receptor LRP5 or 6 (Figure 1-3A). Formation of the Wnt-Fz-LRP complex recruits the scaffolding protein Dishevelled (Dvl), resulting in LRP phosphorylation and activation which recruits Axin. This leads to inhibition of Axin-mediated β-catenin degradation. Without constant degradation, β-catenin accumulates in the cytoplasm and translocates to the nucleus. In the nucleus, β-catenin complexes with the family of T cell factor/lymphoid enhancer factors (TCF/LEF). β-catenin binding to TCF/LEF proteins provides a transcription activation domain. As a result of this complex, Wnt target gene expression is activated [65].

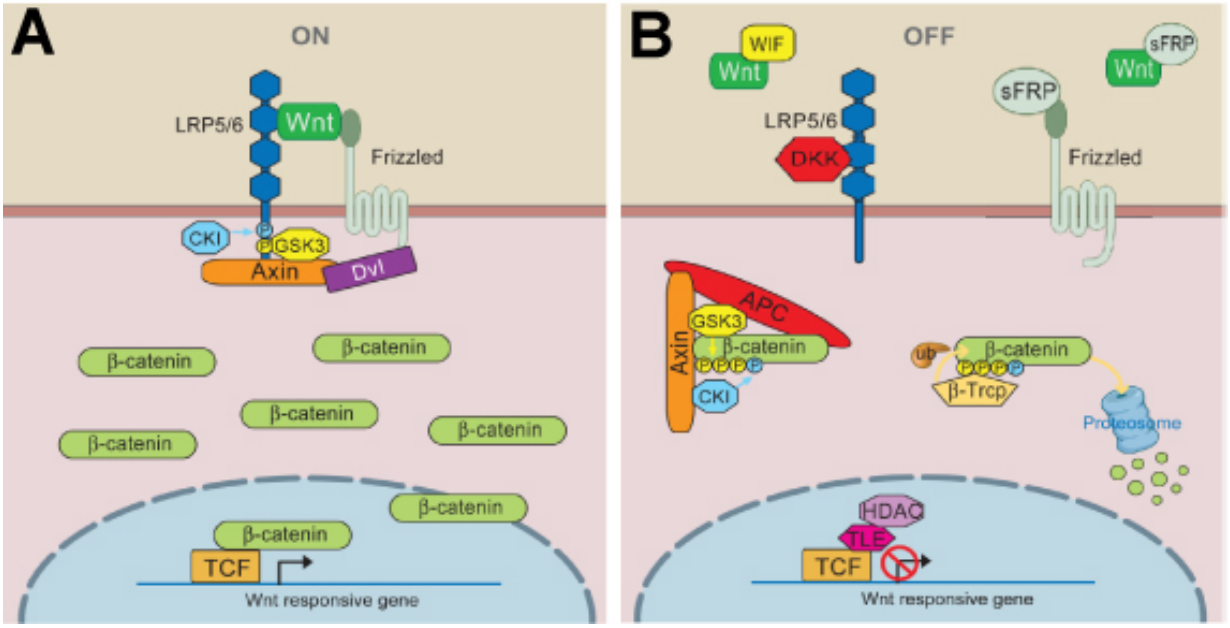

Figure 1-3. Diagram of the Canonical Wnt Pathway [3] (A) Wnts are the primary agonists and form a complex with LRP5/6 and Fz to activate signaling. In the presence of Wnt ligand, a receptor complex forms between Fz and LRP5/6. Dvl recruitment by Fz leads to LRP5/6 phosphorylation and Axin recruitment. This disrupts Axin-mediated phosphorylation/degradation of β-catenin, allowing β-catenin to accumulate in the nucleus where it serves as a coactivator for TCF to activate Wnt-responsive genes. (B) WIF1 and sFRP antagonists bind directly to secreted Wnts and/or Fz binds directly to secreted sFRP. DKK antagonist proteins bind LRP5/6 to prevent Fz-LRP6 complex formation. In the absence of Wnt, cytoplasmic β-catenin forms a multiprotein complex and is phosphorylated by CK1 (blue) and subsequently by GSK3 (yellow). Phosphorylated β-catenin is recognized by the ligase β-Trcp, which targets β-catenin for proteasomal degradation. Wnt target genes are repressed by TCF-TLE/Groucho and HDAC.

In the absence of Wnt (Figure 1-3B), cytoplasmic β-catenin interacts with the adenomatous polyposis coli gene product (APC) as well as Axin scaffold proteins and is a substrate for casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β). CK1 and GSK3β sequentially phosphorylate the amino terminal region of β-catenin, resulting in β-catenin ubiquitination by β-Trcp and proteasomal degradation. Constant elimination of β-catenin prevents it from reaching

[3] Modified from Ref. 65. MacDonald, B.T., K. Tamai, and X. He, *Wnt/beta-catenin signaling: components, mechanisms, and diseases.* Dev Cell, 2009. **17**(1): p. 9-26.

the nucleus; thereby, allowing TCF/LEF to stay bound to and repress Wnt target genes. TCF/LEF factors, when bound to DNA at Wnt-responsive genes, are able to interact with other factors, such as Groucho and histone deacetylase, to repress transcription [70].

Noncanonical Pathway

Noncanonical Wnt signaling pathways are less understood, but appear to function in a β-catenin-independent manner to regulate processes such as convergent extension during vertebrate gastrulation. In vertebrates, the noncanonical pathways have been termed the Wnt/Calcium and Wnt/c-Jun N-terminal kinases (JNK) pathways. In brief, activation of the Wnt/Calcium pathway involves Wnt binding to a Frizzled receptor, leading to the release of intracellular calcium and the activation of enzymes such as Ca^{2+}/Calmodulin-Dependent Protein Kinase II and Protein Kinase C. The Wnt/JNK pathways appear to similarly use Frizzled receptors, Dishevelled, JNK and Rho family guanosine triphosphatases (GTPases). Recent data have also implicated components of noncanonical Wnt pathway in diverse forms of human cancer [71].

There are numerous components of the Wnt network, from the plasma membrane to the cell nucleus. While an understanding of the overall organization of the network is beginning to emerge, much is still unknown. Wnt signals ultimately promote cell proliferation and tissue expansion. Numerous Tcf target genes have been identified in diverse biological systems and include genes involved in cancer, such as cMyc and cyclin D1.

The Wnt signaling pathway is frequently altered in cancer. While no direct mutations of Wnt have been found in cancer, several of the downstream molecules in the Wnt signaling pathway (i.e., β-catenin, APC, Axin) are dysregulated in a variety of human tumors. Probably the most characterized of them are inactivating, germline-mutations in APC found in colorectal cancer. Activation of Wnt signaling is believed to play a role in the progression and pathogenesis

of cervical cancer. It has been shown that β-catenin is increased within the cytoplasm and nucleus in invasive cervical carcinomas. In addition, *in vivo* activation of the Wnt pathway is sufficient to induce transformation of HPV-immortalized human keratinocytes [72].

We focused specifically on Wnt inhibitory protein (WIF1), an antagonist of the Wnt pathway. WIF1 has a unique N-terminal signal sequence, WIF domain (WD), and five epidermal growth factor (EGF)-like repeats which mediate Wnt binding to WIF1 [73]. WIF1 mainly functions as a secreted inhibitor of Wnt signaling by binding to Wnt proteins and thus competing with the binding of Wnt proteins to the frizzled receptor. *WIF1* expression is downregulated in prostate, breast, lung and bladder tumors [74], suggesting that *WIF1* is a potential TSG. Loss of *WIF1* expression in tumors may lead to unrestricted binding of Wnt ligands to the frizzled receptor, followed by enhanced transcription of target genes in the Wnt pathway. Several groups have correlated decreased *WIF1* expression with aberrant promoter hypermethylation. While the *WIF1* gene is a target for epigenetic silencing in some tumor types and its expression is downregulated in several cancers, it is currently unknown if this gene is subject to epigenetic silencing in cervical cancer. To determine if *WIF1* is epigenetically silenced in cervical cancer, we have analyzed the expression and DNA methylation status of the *WIF1* promoter in a panel of cultured human cervical tumor cell lines and surgically-obtained human cervical tumors. Our results demonstrate that *WIF1* hypermethylation is a frequent event in cervical cancer and lend strength to the idea that dysregulation of Wnt signaling is an important contributor of human cervical tumorigenesis.

CHAPTER 2
MATERIALS AND METHODS

Cervical Specimen

All human tissue samples were obtained from patients under protocols approved by Institutional Review Boards at the Moffitt Cancer Center (Tampa, FL). Tumor tissue (n = 22) and adjacent matched normal stratified epithelium (n = 22) were obtained from archived formalin-fixed paraffin-embedded (FFPE) blocks from the tumor repository. The archived tissue came from women diagnosed with squamous cell cervical cancer and having a surgical procedure between 1993 and 1999 at the Moffitt Cancer Center. Cases were restricted to those that did not receive radiation treatment before surgery. Histological diagnosis, tumor stage and grade were reviewed by a pathologist at Moffitt Cancer Center according to the WHO and the international union against cancer (UICC) classification of tumors.

The Pap samples (n = 8) are scrapings of the cervix obtained from patients from four sites: the local Health Department, Tampa General Hospital, Lifetime Cancer Screening and the Moffitt Cancer Center. Routine gynecological examinations of healthy, disease-free women were conducted. Exfoliated cells were placed in 20 mL of PreserveCyt solution, mixed and separated into 4 ml aliquots and stored at -80^{o}C. One milliliter of the solution was transferred to a new tube with a pipette and pelleted. To resuspend the pellet, 200 µl of phosphate-buffered saline (PBS) was added, and DNA was extracted using the QIAamp DNA extraction kit (Qiagen, Valencia, CA). Sample #3 is the only one that is HPV negative. The other seven samples were found to have one or more HPV types: 1, 16, 51, 58, 59 and 62.

Cell Culture and 5-azadC Treatment

The cervical tumor cell lines C-33A, CaSki, HeLa 229 and SiHa were purchased from the American Type Culture Collection and cultured according to their specifications. In short, HeLa,

SiHa and C-33 lines were grown in Eagle's Minimal Essential medium (Mediatech, Manassas, VA) with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Grand Island, NY) and penicillin-streptomycin (Mediatech). The CaSki line was grown in Roswell Park Memorial Institute 1640 medium (Meditech) with 10% FBS. All cell lines were cultured and maintained in monolayers at 37°C in a humidified incubator under 95% O_2 and 5% CO_2. For the drug treatment, cells were seeded, allowed to recover overnight, then 5-azadC (Sigma-Aldrich, St. Louis, MO) was added to complete growth media at a final concentration of 5 μM. Fresh drug was added every 24 hr for 5 d. After treatment, the cells were washed with PBS and allowed to recover in their respective medium for 2 d prior to collection.

RNA and DNA Isolation

One 10 cm plate of confluent cells cultured with and without 5-azadC was harvested for RNA and DNA extraction with 1 mL of TRizol (Invitrogen) reagent for 5 min at room temperature. After phenol/chloroform extraction and phase separation, RNA was digested according to the manufacturer's instructions for on-column DNase I digestion with RNase-free DNase I (Qiagen) and RNAeasy Kit (Qiagen). The DNA was isolated according to manufacturer's instructions from the organic phase of the RNA isolation. The 260/280 nm absorbance ratio and sample concentrations of the RNA and DNA was determined using the NanoDrop ND-8000 (Thermo Scientific, Wilmington, DE).

All genomic DNA isolated from patient samples was conducted at the Moffitt Cancer Center by either centrifugation of 1-4 ml of Thin Prep solutions into a pellet that was resuspended in PBS (Pap samples) or by sectioning tissues from paraffin blocks into small pieces and deparaffinized by xylene extraction. The genomic DNA was then extracted using QIAmp mini DNA Kit (Qiagen) according to manufacturer's protocol. The concentration of the extracted DNA was then determined by the NanoDrop1000 Spectrophotometer (Thermo Scientific).

Sodium Bisulfite Modification

At the Moffitt Cancer Center, sodium bisulfite conversion of 1 µg of patient tissue-derived DNA was carried out with the EZ DNA Methylation-Direct kit (Zymo Research Corporation, Orange, CA), following the manufacturer's protocol. Bisulfite-modified DNA was resuspended in 30 µl of water and stored at –80°C in six 5 µl aliquots to avoid several freeze/thaws. Cell line DNA was bisulfite treated using a specific in-laboratory protocol that consistently yields a 99.8% efficiency of cytosine conversion [75]. The protocol is: the day before bisulfite treatment, distilled water was degassed (dg.dH_2O) by boiling ~200 mL of distilled H_2O (dH_2O) for 20 min in a glass beaker, and carefully poured into a 125 mL bottle until it was completely full (above the lip). The cap was screwed on the bottle tightly and the dg.dH_2O was cooled overnight on the bench top. Immediately prior to bisulfite treatment, 3 N NaOH and 100 mM hydroquinone were prepared by dissolving each chemical in dg.dH_2O. Total genomic DNA (1-2 µg) in 20 µl 0.1X TE was pipetted into a 0.65 ml micro-centrifuge tube (that fits in a thermocycler) containing 10 µl of denaturation buffer (6.5 µL dg.dH_2O, 3.0 µL 3N NaOH, and 0.5 µL 0.5 M EDTA, pH 8.0). Saturated sodium metabisulfite solution was made as follows: to a 20 ml-capacity glass scintillation vial containing a small stir bar, 0.1 ml of 100 mM hydroquinone, 5 g sodium metabisulfite (Sigma, previously aliquoted into air-tight vials), and 7 ml dg.dH_2O were added, and the solution was immediately stirred. Then, 1.0 ml of 3 N NaOH was quickly added. The solution was adjusted to pH 5.0 at room temperature with additional 3 N NaOH and preheated to 50°C in a water bath. A saturated solution was employed to promote sulfonation of cytosine, the first step of bisulfite deamination. Samples of genomic DNA in denaturation solution were heated at 98°C for 5 min with the tube caps open in a thermocycler. With one minute remaining in the denaturation step, the preheated sodium metabisulfite solution

was transferred to a stir-plate and stirred gently. After 5 min denaturation, 0.2 ml of sodium metabisulfite was rapidly added to each sample while in the thermocycler. Samples were capped, vortexed and incubated for 6 hr in a covered water bath at 50°C. Following deamination, the DNA was desulfonated and cleaned using the EZ Bisulfite DNA Clean-up kit (Zymo Research Corporation) according to manufacturer protocol and eluted in 10 µl of M-Elution Buffer.

Polymerase Chain Reaction (PCR) and Pyrosequencing

Following bisulfite modification, a 20 µl PCR with a final concentration of 1× Coral Buffer, 250 nM deoxyribonucleotide triphosphates (dNTPs), 250 nM forward and reverse primer, 1 U of HotStarTaq Plus (Qiagen) and 2 µl of bisulfite-treated DNA was incubated in the thermocycler as follows: 95°C for 5 min, once; 94°C for 30 sec, 59°C for 30 sec, 72°C for 30 sec, repeated 45 cycles. The specific primers used were (Forward, F) 5'-GTA GGT TTT TTG GTA TTT AGG T -3' and (Reverse, R) 5'-CAT ACT ACT CAA AAC CTC CT-3'. A second nested PCR was preformed using 2 µl of the original PCR product in the same reaction mixture. Amplification was checked by agarose gel electrophoresis. A series of controls were used to validate the assay: no template, no sequencing primer, sequencing primer only, sequencing primer with reverse primer only, beads only and a blank well. Also, *in vitro* methylated bisulfite-treated DNA (Zymo Research Corporation) was used as a methylation positive control for all pyrosequencing runs. All samples and controls for each reaction were then analyzed using the PyroMark MD system (Biotage, Charlotte, NC). Methylation density was quantified using the PyroMark Pyro Q-CpG 1.0.9 software.

Reverse Transcriptase PCR (RT-PCR)

Total RNA was used in first-strand complementary DNA (cDNA) synthesis reactions using GoScript Reverse Transcriptase System and random hexamer primers (Promega, Madison, WI). WIF1 transcript levels were subsequently analyzed by PCR. The cDNA WIF1-specific

primers used were (F) 5'-CCG AAA TGG AGG CTT TTG TA-3' and (R) 5'-TGG TTG AGC AGT TTG CTT TG-3'. They were designed to span a gap between exons to ensure amplification of cDNA and not genomic DNA. β-actin served as a control for RNA integrity and was amplified using primers (F) 5'-CCC TGG CAC CCA GCA C-3' and (R) 5'-GCC GAT CCA CAC GGA GTA C -3'. PCR thermocycler conditions for WIF1 were 95°C 5 min, once; 94°C for 30 sec, 59°C for 30 sec, 72°C for 30 sec, repeated 45 cycles. PCR conditions for β-actin mRNA were the same as for WIF1 transcript except that an annealing temperature of 60°C was used and PCR was conducted for 20 cycles. A PCR reaction substituting dH_2O for cDNA was conducted as a negative control.

Methyltransferase Accessibility Protocol for Individual Templates (MAPit)

Nuclei isolation and DNMT probing of nuclei from CaSki, HeLa and SiHa cells were isolated from ~4-7 x 10^6 cells at 4°C under buffer conditions which preserve the integrity of nuclei and chromatin structure. After harvesting, cells were washed twice with PBS and resuspended in cell resuspension buffer (CRB: 20 mM HEPES, pH 7.5, 70 mM NaCl, 0.25 mM EDTA, 0.5 mM EGTA, 0.5% glycerol (v/v), 10 mM DTT, 0.25 mM phenylmethylsulfonyl fluoride). After pelleting by centrifugation at 1000 × *g*, cells were resuspended in cell lysis buffer (CLB: 1× CRB plus 0.19% (v/v) NP-40) for ten minutes on ice. Nuclei were then washed twice with CRB, and a 1-2μL aliquot was stained with 4% (w/v) trypan blue and visualized by light microscopy to confirm their integrity. Nuclei were finally resuspended in methylation buffer (MB; 1× CRB plus 160 μM *S*-adenosylmethionine). After pre-warming nuclei to 37°C for 5 min, 60 U and 100 U of M.CviPI were added for 15 min at 37°C. Methylation reactions were stopped by adding two volumes of methylation stop buffer (MSB; 100 mM NaCl, 10 mM EDTA, pH 8.0, 1% SDS (w/v)), and then incubated overnight with 100 μg/ml proteinase K at 50°C. DNA was

isolated by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and concentrated by ethanol precipitation.

After isolation, DNA was treated with sodium bisulfite as described above. PCR primers designed to amplify a 712 basepair segment of the promoter were designed. Primer sequences were (F) 5'- ATT ATT ATT ATT ATT ATT AGY ATT TAG TT-3' and (R) 5'- CAA RCA CAA AAA AAT RCT CCA AA -3'. Three 20 µl mixtures with a final concentration of 1× Coral Buffer, 250 nM dNTPs, 250 nM forward and reverse primers, 275 nM $MgCl_2$, 1 U of HotStarTaq Plus (Qiagen) and 4 µl of bisulfite-treated DNA were incubated in the thermocycler as follows: 5 min at 95°C, once; 95°C for 30 sec, 53°C for 30 sec, 72°C for 2 min, repeated for 40 cycles. Following PCR, products were digested with restriction enzymes R.HindIII and R.XhoI. After digestion the products were pooled and resolved by an agarose gel electrophoresis. Subsequently, the bands were excised and gel purified using the QiaexII Gel Extraction kit (Qiagen). The digested, purified PCR products were then ligated at 16°C overnight into a R.HindIII- and R.XhoI-digested BlueScript SK minus plasmid (Stratagene, Santa Clara, CA) with T4 ligase (New England Biolabs, Ipswich, MA). Recombinants were identified by colony PCR with Apex Taq (Genesee Scientific, San Diego, CA) in the same reaction mixture as above. The inserts were sequenced at the University of Florida Interdisciplinary Center for Biotechnology Research DNA sequencing core laboratory using a vector-encoded primer (M13-Rev).

Immunohistochemistry

Two tissue microarrays were assembled at Moffitt with 4 µm sections of FFPE tissue sectioned from each cervical tumor with matching normal adjacent tissue. After sections were deparaffinized by xylene and rehydrated through graded alcohols, the samples were blocked using the Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA) following the

manufacturer's recommended protocol. Antigen retrieval by steam heat for 20 min with 10 mM sodium citrate, pH 6.0 was conducted prior to overnight incubation with the rabbit anti-C-terminus primary antibody diluted 1:50 (Abcam, Cambridge, MA). Samples were then washed and incubated with biotinylated-goat anti-rabbit antibody from the Rabbit IgG ABC kit (Vector Laboratories). Detection of the antigen was carried out by avidin-biotin complex following standard procedures. The tissue microarray was developed with peroxidase and 3,3'-diaminobenzidine tetrahydrochloride (reddish-brown reaction product). Sections were then counterstained with haematoxylin to stain the nuclei purple and mounted in an aqueous mounting medium (Fisher). A control section was performed where the primary antibodies was replaced by irrelevant rabbit monoclonal antibody of the same isotype and at the same concentration as the specific primary antibody. Analysis was performed under 100× magnification on a Leica DM6000B microscope (Leica Microsystems, Bannockburn, IL), and images were taken with a Retiga SRV Fast 1394 digital camera (QImaging, Surrey**,** British Columbia, Canada).

CHAPTER 3
RESULTS

WIF1 is Epigenetically Silenced in Cervical Cancer Cell Lines

To determine if the *WIF1* gene is targeted for epigenetic silencing in cervical cancer, we cultured a panel of four cervical cancer lines (C-33A, CaSki, HeLa, and SiHa) in the presence or absence of the DNA demethylating drug 5-azadC. Using RT-PCR we observed in the untreated cells that C-33A, CaSki, and HeLa cell lines expressed varying amounts of WIF1 transcript while SiHa did not. However, when cultured in the presence of 5 mM 5-azadC, the CaSki, HeLa, and SiHa lines displayed elevated levels of WIF1 transcript (Figure 3-1). These data suggest that *WIF1* expression may be epigenetically repressed by DNA methylation in CaSki, HeLa and SiHa cells.

The varied reactions of the cell lines to 5-azadC were of note. The SiHa cell line continued to divide during 5-azadC treatment, while the HeLa cell remained quiescent and quickly divided during the two-day recovery period in the absence of drug. Similarly, CaSki cell numbers remained constant during 5-azadC treatment, but slowly divided afterward. In contrast some C-33A cells died during 5-azadC treatment, and there was little cell division during the two-day recovery period. The difference in response to the drug treatment appeared to correlate with the presence of the HPV genome and inversely correlate with the methylation status at this locus, which may or may not be of significance.

To determine if the difference in *WIF1* expression levels was correlated with promoter DNA hypermethylation, we analyzed the methylation status of *WIF1* by pyrosequencing. Pyrosequencing is essentially ‘sequencing by synthesis’ to quantitatively analyze cytosine

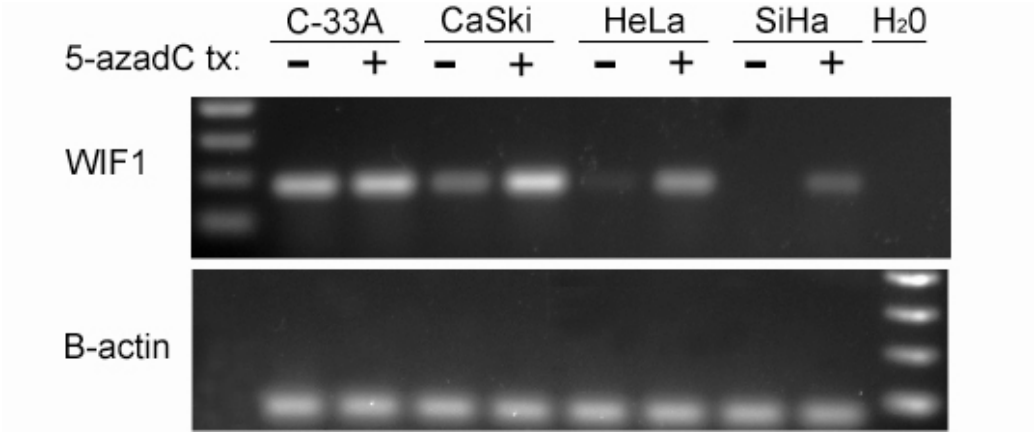

Figure 3-1. RT-PCR of Cervical Cell Lines Before and After 5-azadC Treatment. Cultures of the cervical tumor cell lines C-33A, CaSki, HeLa and SiHa were untreated (–) or treated with 5 µM 5-azadC (+) for 5 d (fresh medium and drug were added every 24 hr). Following this treatment, total RNA and genomic DNA were prepared from the cells. RT-PCR analysis of *WIF1* expression (top panel) and the housekeeping gene β-actin as a control for RNA integrity (bottom panel).

methylation at individual CpG dinucleotides after PCR amplification of a bisulfite-converted sequence. Briefly, bisulfite conversion of unmethylated cytosines in CpG dinucleotides results in conversion of C to U while methylated cytosines remain as C. Pyrosequencing requires PCR of a segment of the gene of interest from bisulfite-converted genomic DNA using primers that amplify in a methylation-independent manner by avoiding areas with CpG present. In addition, one primer is designed with a 5' biotin tag. PCR amplicons are subsequently purified using streptavidin-coated beads, and then denatured to single-stranded DNA. A sequencing primer is annealed to the captured strand and added to a 96 well reaction plate containing DNA polymerase and dNTPs. As nucleotides are incorporated onto the 3' end of the oligonucleotide primer, pyrophosphate (PPi) is given off as a byproduct. The reaction mix also contains ATP sulfurylase which quantitatively converts PPi to ATP in the presence of adenosine 5' phosphosulfate. This ATP drives a luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the ATP concentration. The light

produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) camera and is recorded by the pyrosequencer. Each light signal is proportional to the number of nucleotides incorporated, and thus DNA methylation is quantified at each CpG dinucleotide.

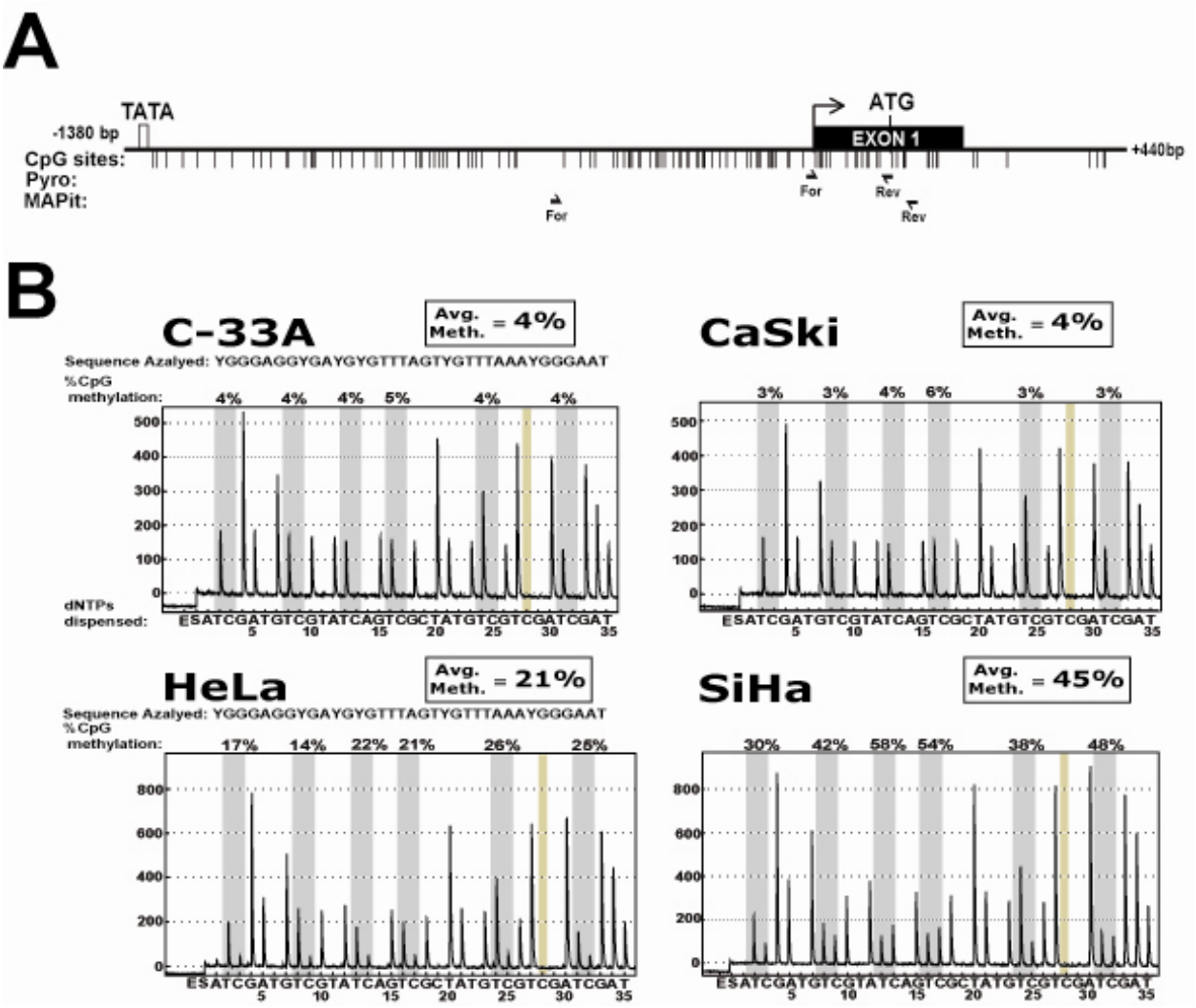

Figure 3-2. *WIF1* Promoter and Pyrograms of Cervical Cell Lines. (A) Schematic representation of the *WIF1* promoter. Each vertical line represents a CpG dinucleotide in the sequence. Positioned below the diagram are the primers used for pyrosequencing and MAPit, respectively. (B) Data output from the pyrosequencer, termed a pyrogram. Shown in the pyrograms are the proportions of nucleotides incorporated along with the sequence of nucleotides dispensed shown on the x-axis. The percent methylation at each CpG dinucleotide (gray rectangles) is shown above the CpG site. Average CpG methylation (Avg. Meth.) within the segment of the gene analyzed is displayed in the box at the top of the pyrogram. There are several quality controls where a nucleotide that should not be incorporated is dispensed (site 6,14, 28 and 29). A bisulfite conversion control dispenses a C directly after a converted T to check the bisulfite conversion efficiency (tan rectangle).

We designed pyrosequencing primers to amplify a 164 basepair region of the *WIF1* promoter. This assay quantitatively measures the first seven CpGs following the transcriptional start site using the forward PCR primer as the sequencing primer (Figure 3-2A). When pyrosequencing was conducted on bisulfite-modified genomic DNA harvested from the four cervical cancer cell lines, we observed that the cell lines that express *WIF1* in the absence of 5-azadC (C-33A and CaSki), exhibited very low levels of DNA methylation (<3%). Conversely, cell lines which expressed little to no *WIF1* in the absence of 5-azadC, HeLa and SiHa cells, exhibited high and moderate levels of methylation, respectively (21% and 45%) (Figure 3-2B). Genomic DNA harvested from HeLa and SiHa cells cultured with 5-azadC displayed a 10-15% decrease in average methylation, while C-33A and CaSki showed little change (Figure 3-3). These data demonstrate that the expression of *WIF1* is correlated to the methylation status of its promoter in cervical cell lines.

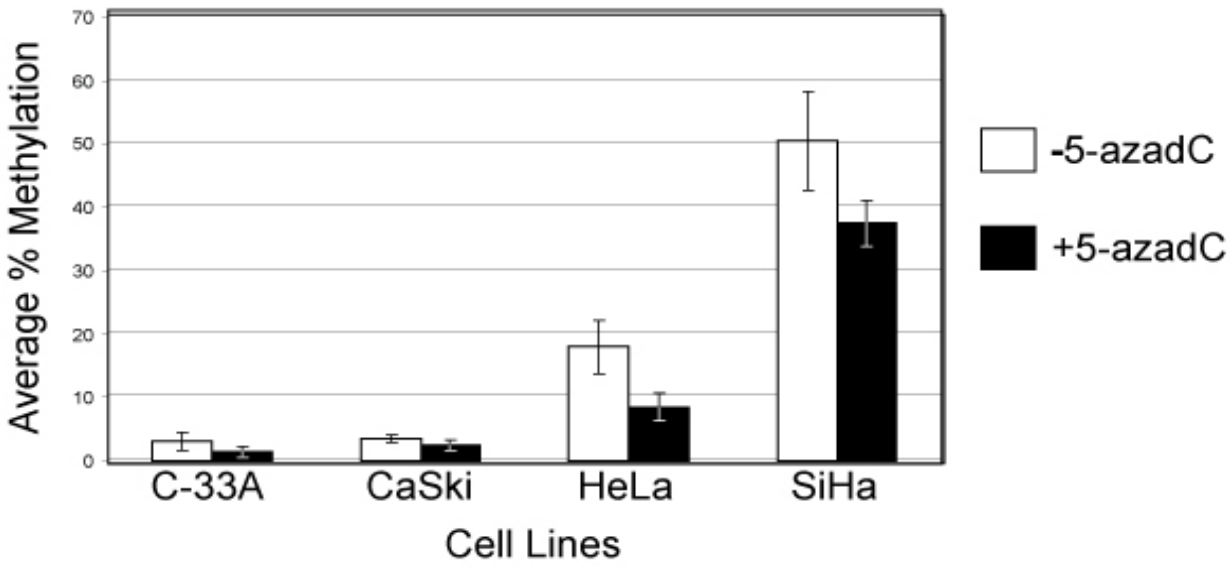

Figure 3-3. Average Methylation of the *WIF1* Promoter Before and After 5-azadC Treatment. Cultures of the cervical tumor cell lines C-33A, CaSki, HeLa and SiHa were untreated (white bars) or treated with 5 μM 5-azadC (black bars). Shown are the averages and standard deviation for the six CpGs assayed by pyrosequencing as outlined in Materials and Methods.

Aberrant Chromatin Footprint in CaSki and SiHa Cell Lines

To determine if promoter methylation might be repressing *WIF1* expression by altering chromatin accessibility, we preformed a methyltransferase accessibility protocol for individual templates (MAPit) on the CaSki and SiHa cell lines. This technique probes chromatin structure with minimal disruption to nuclei integrity to capture an accurate footprint of chromatin structure and nucleosome dynamics *in vivo*. Nuclei were extracted and probed with M.CviPI, a DNA methyltransferase (DNMT) that only methylates GC dinucleotides. Accessibility of DNMTs to GC sites is impaired based on the strength and span of the protein-DNA interaction to be probed. Methylated cytosines that resist chemical deamination are detected positively by the PCR-based technique of bisulfite genomic sequencing. This unique capability to score methylation at multiple sites in single molecules permits detection of inherent structural variability in chromatin structure and non-histone protein-DNA interactions. MAPit analysis was conducted on the active and repressed *WIF1* promoter of CaSki and SiHa lines, respectively (Figure 3-4).

In the CaSki cell line, which expressed *WIF1*, two populations of cells were evident: one population with two distinct areas of methylation and one population with nearly no methylation (Figure 3-4A). After probing with M.CviPI, there were three distinct regions of open chromatin (Figure 3-4B). However, there was no difference in accessibility between the methylated and unmethylated populations. It is of note that sequence inspection revealed two consensus Sp1 binding sites (5'-GGCGGG-3') within the two regions devoid of endogenous methylation and inaccessible to the methyltransferase. This is consistent with other reports showing that Sp1 binding to DNA may be able to protect sequences from DNA methylation [76]. Near the upstream SP1 site, it has also been shown that NF-kβ and MYC-associated zinc finger (MAZ) transcription factors bind [77]. In the SiHa cell line in which *WIF1* was highly methylated and not expressed, high levels of endogenous methylation were observed throughout the body of the

promoter and accessibility to the methyltransferase seemed to vary more, thus suggesting dynamic chromatin reorganization (Figure 3-4C).

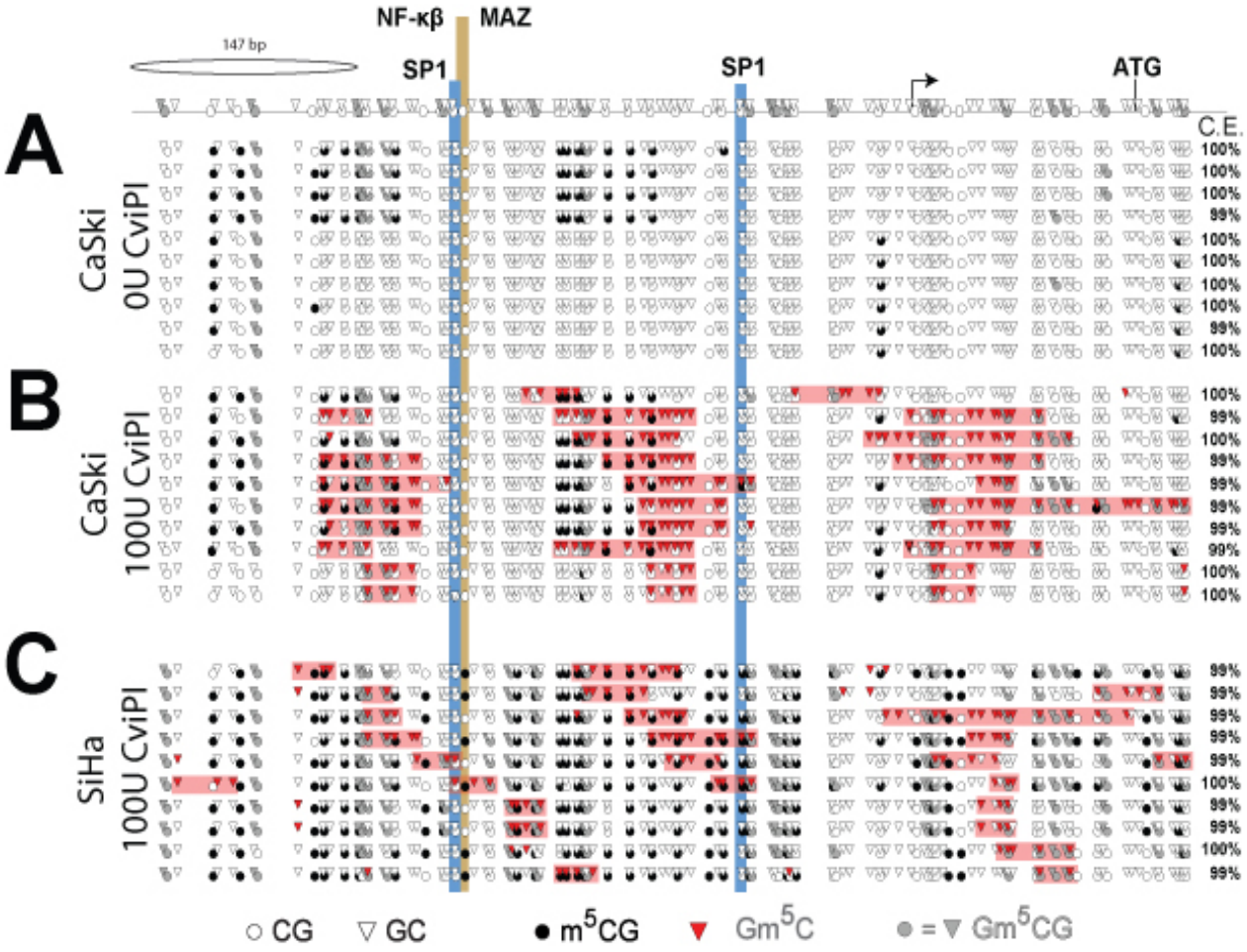

Figure 3-4. Chromatin Probing of CaSki and SiHa Cell Line. Nuclei from both cells lines were isolated and chromatin structure was probed as stated in the Materials and Methods. Endogenously methylated CpG (CG) sites are depicted with black circles and unmethylated CpG with white circles. All GC sites are denoted by triangles, and those that were methylated by M.CviPI probe are red. Regions that were not protected against methylation are indicated by rectangles spanning at least two contiguous GC sites (i.e., one methylated site does constitute accessibility). Methylated GCG (Gm^5CG) sites are indicated in gray and cannot be discerned from endogenous methylation or M.CviPI modified sites. The bisulfite conversion efficiency (C.E.) for each molecule is shown at the right. Transcription factor binding sites for Sp1 (blue) and both NF-κB and MYC-associated zinc finger (MAZ) (tan) are indicated.

WIF1 Promoter is Aberrantly Methylated in Squamous Cell Cervical Tumors

To determine if promoter methylation of the *WIF1* gene occurs in primary cervical tumors, we used pyrosequencing to analyze the methylation of *WIF1* in patient-derived cervical squamous cell carcinoma samples. Matched adjacent normal cervical stratified epithelium and Pap smear samples from different patients were also analyzed. Cervical tumors and normal tissues were obtained from the Moffitt Cancer Center Tissue Bank. DNA was extracted, treated with sodium bisulfite and PCR amplified with the *WIF1* pyrosequencing primers. PCR amplicons where then analyzed with the PyroMark MD and Pyro Q-CpG software. All cases samples were re-analyzed with repeated PCR and pyrosequencing reactions to validate the initial pyrosequencing findings. Subsets of bisulfite-treated samples were also analyzed multiple times by bisulfite genomic sequencing, with analogous results (data not shown).

To ascertain if aberrant methylation of the *WIF1* promoter occurs in cervical cancer, we pyrosequenced 22 high-grade squamous cell carcinomas. Of these 22 tumors, only 17 produced PCR amplicons of sufficient quality for pyrosequencing. We grouped these specimens into three categories based on their average methylation of six CpG sites as followed: little or no methylation (0-9%), moderate methylation (10-19%) and heavily methylated (>20%). Of the 17 tumor samples, 6 were not methylated, 3 had moderate methylation, and 8 were heavily methylated (Figure 3-5).

We also analyzed a set of 8 Pap smear samples that had been previously genotyped and 7 of which were found to be HPV positive. We found no significant methylation, <5%, in all 8 of the Pap smear samples (Figure 3-6). In addition we analyzed the paired adjacent normal tissue from each tumor sample. Unfortunately, several of theses samples failed to

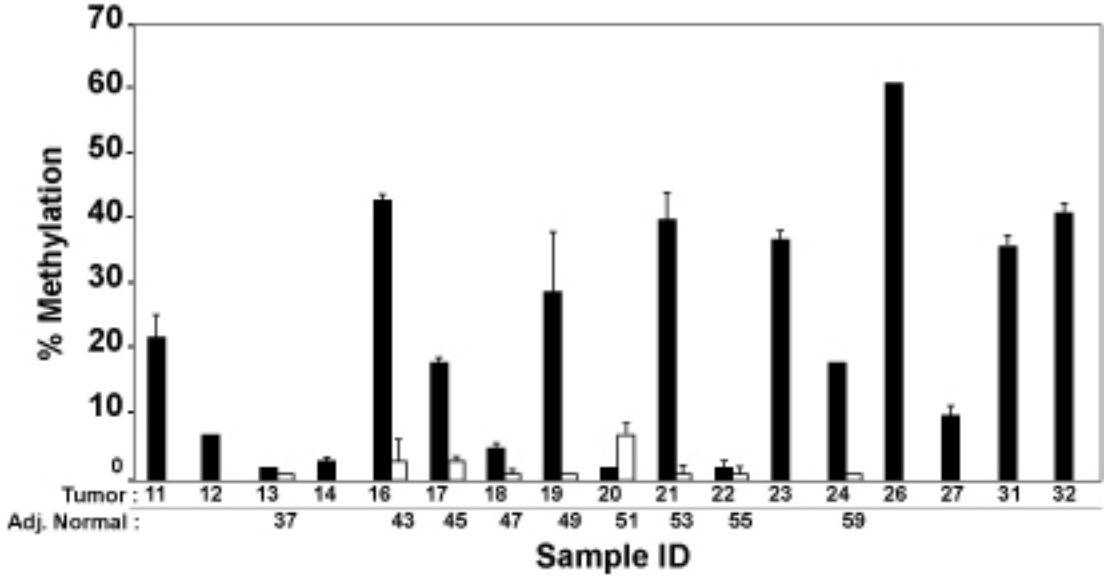

Figure 3-5. Average CpG methylation of Cervical SSC and Adjacent Normal Tissue at *WIF1*. The tumors and adjacent matching normal tissues were given arbitrary numbers. Both the PCR and the pyrosequencing were run in duplicate, and the average methylation status of the *WIF1* promoter is shown along with the standard deviation for each sample. Black bars represent the methylation of the tumor samples and the white bars show the methylation of the paired normal stratified epithelium.

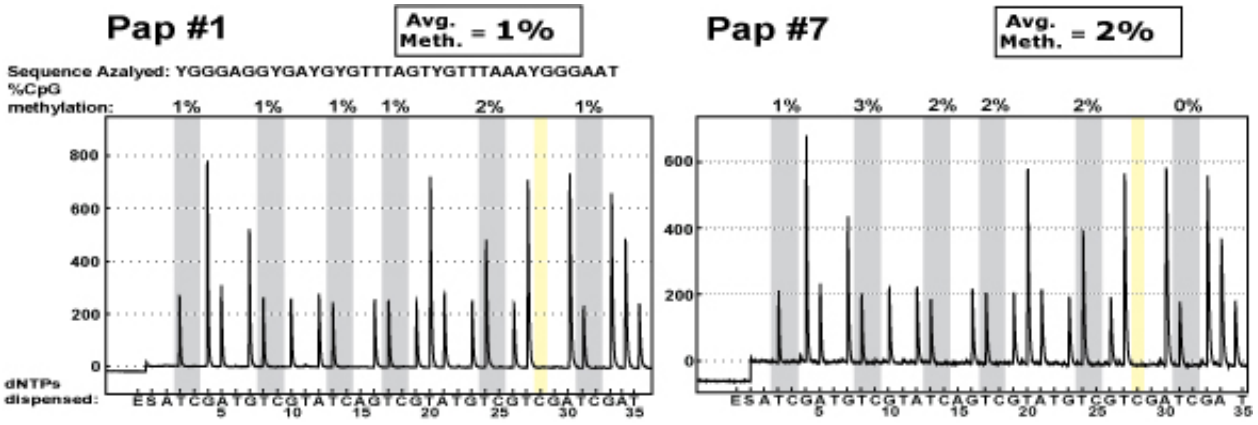

Figure 3-6. Pyrograms of Pap Smear Samples at *WIF1*. The percent methylation at each CpG dinucleotide (gray rectangles) is shown above the CpG site. Average CpG methylation (Avg. Meth.) within the segment of the gene analyzed is displayed in the box at the top of the pyrograms. Seven additional pap smear samples were analyzed with similar results (data not shown).

yield usable amounts of DNA, and we were not able to assess *WIF1* promoter methylation.

However, the 9 matched normal samples that were analyzed all had little or no methylation

(<5%) (Figure 3-5). These data show that this region of the *WIF1* promoter is essentially

methylation-free in normal cervical tissue and supports the notion that the DNA methylation that we observed in the tumor samples resulted from the disease and was not part of the normal biology of cervical tissue.

WIF1 Methylation is not Correlated with WIF1 Protein Levels

To assess if the methylation status of the *WIF1* promoter correlated with the expression of WIF1 protein, we conducted immunohistochemical staining on a tissue microarray including 22 tumors and their adjacent normal tissue. Immunohistochemistry (IHC) is a method for demonstrating the presence and location of proteins in the context of intact tissue. IHC staining is accomplished with antibodies that recognize the target protein. The antibody-antigen interaction is visualized using chromogenic detection, in which an enzyme conjugated to the antibody cleaves a substrate to produce a colored precipitate, in this case brownish-red, at the location of the protein.

Immunostaining of normal tissue with anti-WIF1 antibody indicated that the protein was expressed in the stratified squamous epithelium of the ectocervix. No staining of the underlying cervical parenchyma (Figure 3-7 A1) was observed, except within the endometrial cell layer of blood vessels, which stained intensely with anti-WIF1 antibody. Close examination of the staining indicated a gradient of WIF1 expression within epithelial tissue (Figure 3-7A). Specifically, nearly no staining was observed in the basal epithelium or underlying basement membrane (Figure 3-7A, zones 1 and 2). However, strong staining was evident within the peribasal epithelial layer (Figure 3-7A, zone 3). We observed notable extracellular staining within the layers of differentiated non-keratinized epithelium with diminished staining in cells at the apical surface of the ectocervix (Figure 3-7A, zone 5). A negative control for background staining was incubated with only rabbit IgG and demonstrated that there was no background staining of the WIF1 antibody within the cervical epithelium (Figure 3-7 B).

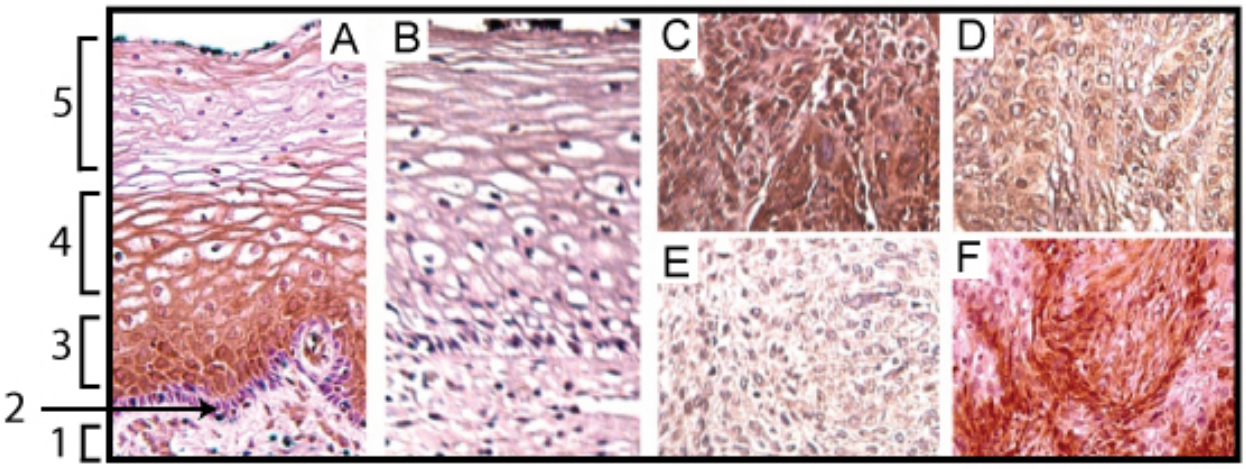

Figure 3-7. WIF1 Immunohistochemistry of Normal Cervical Tissue and SSC. Normal cervical epithelium incubated with (A) anti-WIF1 rabbit antibody or (B) rabbit IgG. No staining from rabbit IgG and the location of WIF1 staining in the cervical epithelium was observed. (C) Tumor section with intense staining and low DNA methylation. (D) Tumor section with mild staining and low methylation. (E) Tumor section with light staining and high methylation. (F) Heterogeneous tumor with a mixed population of stained and unstained cells. Bracketed at the left are the basal epithelium (or underlying basement membrane, zones 1 and 2), peribasal epithelial layer (zone 3), epithelial layer (zone 4) and apical surface of the ectocervix (zone 5).

When IHC was conducted on samples of invasive squamous cell carcinoma (SCC) with an anti-WIF1 rabbit antibody, we observed intense staining (Figure 3-7C) in tumor samples that had little methylation, such as sample 20 for which pyrosequencing showed an average CpG methylation of 2%. However, there was a notable lack of staining within two samples, and this was independent of the methylation status of the *WIF1* gene promoter. For example, little staining was seen in tumor sample 13 (Figure 3-7D) that possessed a mean methylation value of 2%. Moreover, the majority of methylated tumors did not express or had mixed expression of *WIF1*. For example, tumor sample 32, which possessed a high level of *WIF1* methylation (41%), also showed no clear indication of expression of WIF1 protein (Figure 3-7E). We also observed a number of tumors with heterogeneous population of cells that possessed mixed staining for WIF1 within the same lesion (Figure 3-7F), which is a common feature of solid tumors. Of note,

we observed more intense WIF1 staining in less de-differentiated SCC lesions. These studies show that low or absent WIF1 expression is commonly observed in invasive SCC of the cervix, but that this tumor feature may occur independent of *WIF1* promoter methylation status. These findings also suggest that mechanism(s) other then promoter hypermethylation may serve to inhibit *WIF1* expression. We conclude that hypermethylation of the *WIF1* gene is a common event in cervical squamous cell carcinoma but that promoter methylation is not the only necessary cause of its silencing.

CHAPTER 4
DISCUSSION

The rationale for examining DNA methylation at the *WIF1* promoter in cervical cancer was based on three primary factors. First, exploitation of the Wnt pathway is a common mechanism of tumorigenesis. Uncontrolled β-catenin signaling, either from disruption of function of its regulatory proteins (i.e., Axin, APC, WIF1) or genetic mutations that prevent β-catenin degradation, leads to excessive proliferation that predisposes cells to tumorigenesis [65]. Second, *WIF1* expression has been shown to be downregulated in a variety of cancer types, such as bladder, breast, lung and prostate [74]. *WIF1* downregulation is an epigenetic event correlated to promoter hypermethylation in many of these cancers. Third, while some studies have examined the epigenetic regulation of *WIF1* and other genes encoding molecular components of the Wnt signaling pathway, no research has examined *WIF1* silencing in cervical carcinomas.

Using both cervical cancer cell lines and primary formalin-fixed/paraffin-embedded squamous cell carcinomas samples, we established that the *WIF1* gene is commonly subjected to DNA hypermethylation in cervical tumors. *In vitro* studies in cervical cell lines indicated that *WIF1* expression correlated with promoter methylation. Furthermore, we showed that the DNA demethylating agent 5-azadC increased *WIF1* expression. In an effort to show that *WIF1* expression is epigenetically silenced in primary tissue, we conducted pyrosequencing and immunostaining on cervical tumor samples. We found that the *WIF1* promoter is unmethylated and WIF1 protein is expressed in the peribasal and the less differentiated epithelial layers of normal cervical epithelium. In contrast, within high-grade squamous cell carcinomas, WIF1 protein expression was often low to nonexistent. This loss of WIF1 protein expression correlated with promoter hypermethylation in several of the tumors; however, loss of WIF1 protein expression in two of the tumors could not be attributed to *WIF1* promoter hypermethylation. This

suggests that *WIF1* is epigenetically silenced in cervical cancer, but other mechanisms in addition to promoter hypermethylation may be involved.

Lack of complete concordance between *WIF1* promoter hypermethylation and lack of protein expression could be due to several factors. First, pyrosequencing analyses revealed the six CpG sites in the *WIF1* promoter in CaSki cells were unmethylated; however, transcript levels increased after treatment with 5-azaC as assayed by RT-PCR. These findings suggest that the *WIF1* gene is epigenetically silenced in these cells, but by methylation of CpG sites other then the six downstream of the TSS we assayed by pyrosequencing. This is supported by MAPit methylation footprinting experiments, which showed two areas of endogenous methylation further upstream of the TSS in CaSki cells. PCR using the MAPit primers was attempted on several of the tumor samples without success. This failure is likely because FFPE- and bisulfite-treated DNA is very fragmented and thus unable to yield large PCR products. One method to examine if the tumor samples had similar patches of methylation to the CaSki cell line further upstream of the TSS would be to create primers specific for the areas of interest that yield smaller PCR products.

Pyrosequencing of small bisulfite-converted amplicons at the two upstream methylated regions is needed to test the extent to which hypermethylation correlates with *WIF1* downregulation in the primary tumor samples. Methylation of either of these upstream regions correlating with the loss of *WIF1* expression as determined by IHC would lend more support to the hypothesis that the *WIF1* gene is directly epigenetically silenced in cervical cancer.

HPV may also play a role in the epigenetic silencing of the *WIF1* promoter in cervical cancer. This is based on the observation that the three cell lines (CaSki, SiHa and HeLa) that displayed varying levels of *WIF1* epigenetic silencing were all derived from HPV-infected

specimens. By contrast, the cell line derived from a non-HPV-infected specimen did not appear to be epigenetically silenced. HPV viral proteins can directly effect DNA methylation by binding to and recruiting DNMTs [57]. Knockdown of expression of the viral E6 and E7 genes may help to understand if the virus plays a role in *WIF1* hypermethylation. Furthermore, HPV genotyping of the patient cervical tumors should be done to look for a correlation between *WIF1* expression and the presence of HPV.

MAPit footprinting of the CaSki and SiHa cell lines may yield insight into how *WIF1* silencing might occur regardless of promoter methylation. In the *WIF1*-expressing CaSki cells, we observed a region of protection against methylation by M.CviPI just upstream of the transcriptional start site. We hypothesized that this area would be nucleosome free and hence accessible to the DNMT, as has been observed at other transcriptionally-active promoters. The size of the inaccessible region is consistent with a nucleosome (~147 base pairs) that occupies a variety of positions. The variable location of the protected region on each molecule seems inconsistent with protection of the region against methylation by a site-specific transcription factor bound to DNA, such as SP1. It is possible that SP1 or another transcription factor is forming a ternary complex with a nucleosome that occupies several positions.

Interestingly, two different populations of molecules with distinct endogenous DNA methylation patterns were observed in the CaSki cell line. In one group, nearly no endogenous methylation was observed, whereas the other group had two distinct areas of endogenous methylation straddling the upstream SP1 site. Despite these two patterns of methylation by endogenous enzymes, there appears to be little difference in accessibility to M.CviPI between them. This suggests that whatever is bound to the DNMT accessible area binds regardless of the methylation status of the gene. It should be noted, however, that we have only examined a

limited number of molecules from one experiment and there exists the possibility the molecules shown were selectively sequenced due to PCR or cloning bias. Similarly, the heavily methylated *WIF1* promoter in SiHa cells showed clear regions of M.CviPI accessibility but with greater variability (Figure 7C). Often, areas of heavily methylated DNA are inaccessible to the methyltransferase and unable to provide clear footprints. This suggests that, whereas the *WIF1* promoter is heavily methylated in the SiHa cell line, it is not in a fully compacted heterochromatin state.

Further experiments are needed to clarify what is binding to the area upstream of the *WIF1* TSS. Knockdown experiments of the various transcription factors, particularly SP1, in the cell line followed by MAPit might help to clarify what is binding to the DNA. It would be of interest to see if knockdown of SP1 alters *WIF1* endogenous methylation or chromatin accessibility, particularly in the highly methylated SiHa cell line. However, preferential association of NF-κB or MYC-associated with the *WIF1* promoter in Caski, but not in SiHa, cells may explain the difference in expression between the cell lines. Chromatin immunoprecipitation experiments probing for histones might help to create a clearer picture of regulation of the *WIF1* promoter and establish if there is a bias in the MAPit footprint. Better understanding of the *WIF1* promoter region and its associated proteins could potentially lead to use as a cervical cancer drug therapy.

In addition to technical limitations that may have precluded detection of DNA methylation in cervical tumors lacking WIF1 protein expression, it is also possible that non-epigenetic factors are also responsible for loss of *WIF1* expression in these tumors. For example, chromosome rearrangements involving 12q13-15, where *WIF1* is located, are frequent occurrences among tumors. While no DNA mutations of *WIF1* have been reported, one group demonstrated salivary gland pleomorphic adenoma expressing an high mobility group AT-hook 2 (HMGA2)/WIF1

fusion transcript, which resulted in re-expression of wild-type HMGA2 transcripts and very low levels of *WIF1* expression [78]. An alternative explanation for the lack of correlation between *WIF1* promoter methylation and protein expression is that *WIF1* transcription may require an additional activator which could be inhibited or after chromosome rearrangement the gene may be excised. These are a few alternative mechanisms aside from epigenetic silencing that could result in downregulation of WIF1 protein in cervical cancer.

A common aim of TSG research is to evaluate the possibility of a gene being used as either a diagnostic or prognostic tool. We showed that the *WIF1* gene is aberrantly methylated in high-grade squamous cell carcinomas of the cervix, which makes it a potential prognostic marker. However, we failed to obtain any low-grade or mildly dysplastic cervical squamous cells for analysis within the confines of the study. Examining the status of *WIF1* expression at pre-cancerous stages or within low-grade carcinomas may help determine when during tumor progression aberrant methylation of the *WIF1* promoter occurs, and if hypermethylation is an indicator of high-grade squamous cell carcinoma. There exists a possibility that a correlation between expression of *WIF1* and the pathological state or grade of the tumor could be made to provide insight into *WIF1* being a possible biomarker for diagnosis.

It is clear that cancer cells undergo global changes in DNA methylation that lead to chromatin alterations involving the whole epigenome and that entire pathways relevant to cell division are subject to epigenetic dysregulation. As the role of epigenetics in cancer becomes clearer new approaches to cancer prevention, detection, and therapy will begin to emerge.

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BIOGRAPHICAL SKETCH

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