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*WNT5A*, a member of the WNT family of secreted proteins, activates the non-canonical WNT pathway, regulates developmental events and is involved in tissue homeostasis. Misregulation of *WNT5A* has been associated with various types of cancer including pancreatic, colorectal, breast, lung and osteosarcoma. Recent studies have shown that *WNT5A* expression in cancer cells involves non-genetic (epigenetic) changes. The *WNT5A* gene has two similar transcription start sites (termed promoter A and promoter B in this study) and transcription from these sites give rise to two different messages that encode different protein isoforms. Epigenetic studies to date have focused on the *WNT5A* promoter A, however our preliminary analysis found that promoter B transcripts are nearly absent in osteosarcoma cells, but present in normal osteoblasts. The goal of this study was to determine if the decrease in promoter B transcripts in osteosarcoma was due to DNA methylation. We identified 6 CpG islands in the *WNT5A* intron 1 region which contains promoter B and exon 1B, and compared them with data available in NCBI epigenomics website. The NCBI database was also screened for data on the methylation status of these islands in various other cell lines and cancer types. The database analysis shows that there is little or no methylation of Regions 1 – 6 in cells, that normally express *WNT5A* (fibroblast, chondrocytes, and mesenchymal stem cells). However, in some colorectal tumor tissue there is extensive methylation within intron 1. In adjacent normal colon mucosa tissue, Regions 3 and 4 showed some degree of

methylation. Methylation status of the CpG islands in normal osteoblasts and osteosarcoma (SaOS-2) was determined using Sodium bisulfite sequencing. The sequencing data showed that CpG Regions 1 and 2 are unmethylated. Regions 3, 4 and 5 are completely methylated and Region 6 is partially methylated. In normal osteoblasts all 6 regions were unmethylated. A demethylation experiment using 5-Aza-cytidine was performed to determine if decrease in methylation would increase promoter B transcripts. Treatment of SaOS-2 cells with 1 $\mu$ M 5-Aza-cytidine resulted in a 120 fold increase of promoter B transcripts. The methylation data of SaOS-2 cells treated with 1 $\mu$ M 5-Aza-cytidine shows that CpG Region 6 is more prone to demethylation when compared to CpG islands 3, 4, 5. Two clones of Region 6 showed that 4 out of 5 CpG's analyzed were demethylated. 5 clones of Region 3 and 4 showed that only 1-2 CpG's were demethylated out of 24 CpG's in Region 3 and 27 CpG's in Region 4. Overall these results suggest that promoter B transcription is down regulated by DNA methylation of CpG Region 6 and Region 6 is more prone to demethylation compared to Regions 3, 4 and 5.

ROLE OF DNA METHYLATION IN WNT5A PROMOTER B EXPRESSION IN  
OSTEOSARCOMA CELLS

by

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Approved by

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Committee Chair

To my family and friends, for their encouragement and support to help me pursue my dreams.

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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## CHAPTER I

### INTRODUCTION

#### *WNT5A Gene Structure*

The human *WNT5A* gene is located on chromosome 3, band p14.3, between 55,499,744 and 55,521,331 base pairs (Entrez; Gene ID 7474). Alternative splicing and different transcription start sites generate multiple transcripts from the *WNT5A* transcription unit. (Ensembl; Figure 1). In humans, 6 transcripts are generated leading to 6 proteins. One transcription unit has 5 exons and 4 introns and produces an mRNA of 6042 nucleotides (Ensembl; ENST00000264634, Figure 1). The protein derived from this transcript has 380 amino acids. This transcript is derived from what will henceforth be referred to as promoter A and is the most well characterized of the *WNT5A* transcripts. Another transcript is derived from a promoter referred to as promoter B, located in intron 1 of the promoter A transcript (Ensembl; ENSG00000114251, Figure 1). This transcript produces a 1299 bp transcript and a protein of 365 AA. As such, the promoter B protein transcript lacks the first 15 amino acids of the promoter A protein. This project is focused on the genomic sequences associated with the *WNT5A* promoter B. These two transcripts were chosen because *WNT5A* promoter A and promoter B proteins are the only *WNT5A* proteins present in mouse.

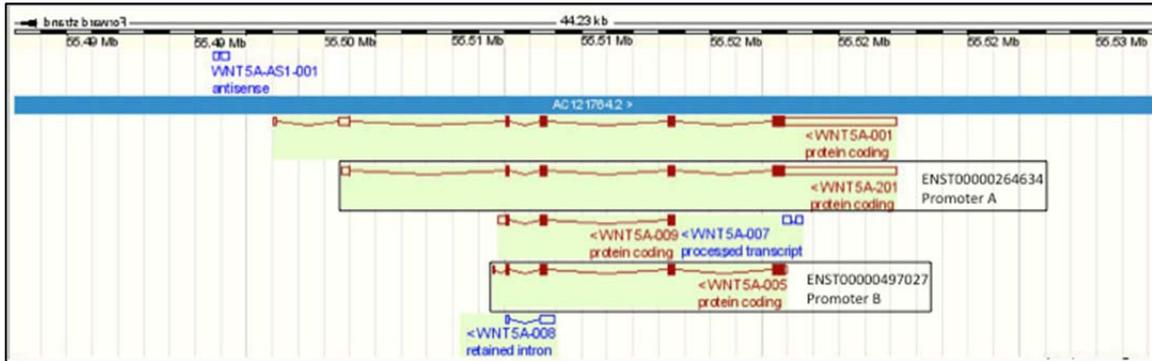


Figure 1. Human WNT5A Transcripts. The two transcripts and associated promoters that will be studied are boxed. The ENST00000264634 transcript correlates with promoter A. The ENST00000497027 transcript correlates with promoter B.

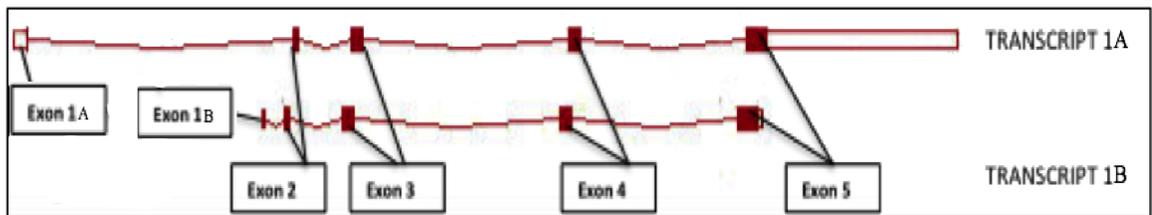


Figure 2. *WNT5A* transcripts 1A and 1B. Exon 1B is located in the intron of transcript 1a. Transcript 1a is derived from promoter A. Transcript B is derived from promoter B.

Promoter A and promoter B give rise to distinct primary transcripts (Figure 2). cDNA clones have been isolated for promoter A (NM\_003392) and promoter B (AK290869), which supports transcription from these alternate start sites. The intron-exon structure of the promoter A and promoter B transcripts are compared in Figure 2. The promoter A transcript includes a unique exon 1A, whereas the promoter B transcript includes a unique exon 1B. Both transcripts share exons 2, 3, 4 and a portion of 5. The promoter B transcript has a much smaller 3' untranslated region (UTR).

### *WNT Signaling and WNT5A*

WNT5A is an extracellular secreted glycoprotein and a member of the WNT family, which plays a role in development, proliferation, differentiation, movement and apoptosis (reviewed in Kikuchi *et al.* 2012). WNT ligands are hydrophobic signal peptides that take part in cell signaling through the Frizzled transmembrane receptor proteins. Wnt signaling acts through two pathways: canonical and non-canonical. The canonical (beta-catenin dependent) pathway is activated when the WNT ligand binds to the Frizzled receptor and the low-density lipoprotein related protein (LPR) co-receptor. This results in a hypophosphorylated beta-catenin, which is translocated into the nucleus. The nuclear beta-catenin binds to the lymphoid-enhancer-binding factor/T-cell-specific transcription factor (LEF/TCF) and activates target gene transcription (Vidal-Puig *et al.* 2008; Nishita *et al.* 2010).

The non-canonical (beta-catenin independent) pathways include the planar cell polarity (PCP)/ convergent extension (CE) pathway (PCP/CE pathway) and the Ca<sup>2+</sup> pathway (Imagawa *et al.* 2008), though the downstream signaling path way is yet to be fully understood. WNT5A signals through the non-canonical pathways by binding to the receptor kinase-like orphan receptor (Ror) 1/2 membrane receptor or different Frizzled receptors and activating RhoA and Rac, PCP/CE pathway and Ca<sup>2+</sup> pathway increasing calcium Ca<sup>2+</sup> ions, leading to effector protein kinase activation (Binder *et al.* 2007). The non-canonical pathway has been shown to have an antagonistic effect on the canonical pathway but recent studies have shown that *WNT5A* acts through canonical pathways as

well (Silver *et al.* 2009; Nishita *et al.* 2010). The consequence of WNT pathway activation/inhibition by WNT5A includes changes in cell migration, cell polarity, differentiation and proliferation (Kikuchi *et al.* 2012).

### *WNT5A, Development and Differentiation*

WNT5A is involved in critical developmental events. When WNT5A was functionally deactivated in mice, their intestines were shortened and formed a bifurcated lumen instead of a single long tube and cell proliferation was also disrupted (Cervantes *et al.* 2009). In another study, an embryonic mouse knockout had truncated limbs and underdeveloped digits due to decreased proliferation of mesenchymal progenitor cells (Yamaguchi *et al.* 1999). Overexpression of WNT5A during early development was also shown to affect cell migration during avian gastrulation by disrupting the non-canonical Wnt signaling pathway (Hardy *et al.* 2008).

WNT5A more recently has been found to be involved in brain development and nerve cell differentiation. A *WNT5A* knockout mouse displayed defects in midbrain morphogenesis such as a rounded ventral cavity and impaired elongation of the midbrain (Andersson *et al.* 2008). WNT5A expression affected D1x homeogene expression, which promotes differentiation of interneurons (Paina *et al.* 2011). And, in another study of *WNT5A* knockout mice, the density of dopaminergic neurite and axon outgrowth was disrupted (Blakely *et al.* 2011).

Most relevant to this study, WNT5A was found to be a regulator of mesenchymal stem cell differentiation into osteoblasts, preadipocytes or myoblasts (Vidal-Puig *et al.* 2008). In a more recent study, the absence of *WNT5A* stopped ontogenesis from human mesenchymal stem cells, whereas its presence inhibits the formation of preadipocytes from human mesenchymal stem cells (Bilkovski *et al.* 2010). Expression of WNT5A was also shown to be necessary for osteoblast and chondrocyte differentiation in mouse endochronal skeletal morphogenesis (Wu *et al.* 2003). *WNT5A* was found to coordinate proliferation and differentiation of chondrocytes by regulating cyclin D1 and p130 expression (Yang *et al.* 2003).

#### *WNT5A and Cancer*

*WNT5A* expression is altered in various types of cancers including osteosarcoma, lung, thyroid, leukemia, colorectal and skin (reviewed in Katoh and Katoh, 2009). *WNT5A* appears to function as both an oncogene and tumor suppressor gene. In several cancers, such as colon and breast cancer, *WNT5A* is downregulated (Ying *et al.*, 2008; Leris *et al.*, 2005), suggesting it acts as a tumor suppressor, conversely, it was found to be overexpressed in some gastric and pancreatic cancers (Kurayoshi *et al.*, 2006; Ripka *et al.*, 2007), suggesting it was acting acting as an oncogene. Weeraratna *et al.* (2002) examined the role of *WNT5A* in melanoma metastasis by transfecting *WNT5A* expression vectors into melanoma cells. These *WNT5A* overexpressing cells showed an increase in protein kinase C (PKC) activity, a protein that plays a role in cell motility and

cytoskeleton changes. In that study, Frizzled-5/*WNT5A* interaction was also disrupted by using an antibody against Frizzled-5. The cells treated with the antibody showed decreased motility in the Boyden chamber invasion assay and the cell movement scratch assay. A decrease in PKC activity was also detected. Overexpression of *WNT5A* has also been shown to play a role in metastasis in pancreatic cancer (Ripka *et al.*, 2007). These studies show a strong association between cell movement and increased *WNT5A* expression during metastases.

#### *WNT5A and Osteosarcoma*

*WNT5A* plays a critical role in the development of osteoblasts and osteoclasts. In one study, formation of osteoclasts and osteoblasts (osteoclastogenesis) was enhanced by *WNT5A* binding to the Ror 1/2 and activation of a non-canonical WNT signaling pathway. This study also showed that osteoblasts cells express *WNT5A* and that its deficiency leads to impaired osteoclastogenesis (Maeda *et al.* 2012). Another study also revealed that overexpression of *WNT5A* enhanced osteoclast differentiation and the non-canonical *WNT5A* signaling accelerates loss of bone mineral density in HIV infected individuals (Santiago *et al.* 2012).

Since these studies have shown the importance of the *WNT5A* signaling pathway in osteoclastogenesis, it would be expected that *WNT5A* has a role in osteosarcoma. Osteosarcoma is a malignant bone tumor, which usually develops during a period of rapid growth that occurs in adolescence. It occurs most commonly in larger bones and in areas

of bone with the fastest growth rate. In one study, the WNT5A signaling in osteosarcoma cells was shown to give these cells some of their metastatic invasive properties (Enomoto *et al.* 2009). In agreement, high levels of the WNT5A were detected in patients with severe osteosarcoma and thus might have a role in its occurrence and progression (Lu *et al.* 2012). Nakano *et al.* (2003) did a microarray study in which they compared the expression of 637 cancer-related genes to detect differential expression between high and low metastatic sublines of osteosarcoma. They found that *WNT5A* was one of the genes associated with adherence, invasiveness and motility of these cells. In a study conducted by Ren *et al.* (2011), they showed that suppression of WNT5A expression leads to inhibition of in vitro cell motility and invasiveness. Thus, *WNT5A* plays an important role in the occurrence and metastasis of osteosarcoma.

### *DNA Methylation*

A single human cell contains a full complement of genetic information but depending on the function of the cell not all genes are expressed. Gene expression and regulation play a crucial role in controlling cell type, cellular functions, development, cell cycle, and tissue homeostasis. Epigenetics is the study of heritable changes in gene regulation and expression without changing the underlying genetic sequence. One epigenetic mechanism is DNA methylation.

DNA methylation is the addition of a methyl group on the position 5 carbon of cytosine. This methylation occurs in CpG dinucleotides (Cytosine-phosphodiester bond-

Guanine) and the methyl groups lie in the major groove of the DNA. When both strands of the DNA are symmetrically methylated they are near each other in the same direction. CpG dinucleotides are usually grouped together in CpG islands. CpG islands are regions around 200 base pair long with a GC content of more than 50%.

DNA methyl transferases (DNMT's) are enzymes responsible for the addition of the methyl groups. There are three types; DNMT1, DNMT3a and DNMT3b. DNMT1 is responsible for the methylation of hemimethylated DNA. When DNA undergoes replication the parental strand retains the methyl groups and DNMT1 is responsible for matching those methyl groups on the newly synthesized daughter strands. DNMT3a and DNMT3b are responsible for de novo methylation of DNA during early development.

The addition of the methyl groups can cause transcriptional repression by either inhibiting proteins from binding to the DNA or serving as a site for binding of proteins with repressive functions. There are families of proteins that recognize methylated DNA (reviewed in Prokhortchouk *et al.* 2008). One family of proteins has a related methyl binding domain (MBD) and includes MBD1, MBD2, MBD4 and MeCP2 proteins. The second family includes zinc finger proteins that bind to the methylated DNA and contains the proteins Kaiso, ZBTB4 and ZBTB38, all of which affect transcription. A third family of related proteins includes UHRF1 and UHRF2, which may have a role in methylation dependent transcriptional regulation (Prokhortchouk *et al.* 2008).

### *WNT5A Epigenetic Alterations*

Recent studies have shown that *WNT5A* misregulation in cancer cells involves non-genetic (epigenetic) changes rather than genetic ones, such as gene mutation and structural rearrangements. DNA hyper or hypomethylation of the *WNT5A* gene regulatory region have been detected in various cancers.

The promoter region of *WNT5A* was found to be hypermethylated and epigenetically silenced or down regulated in esophageal squamous cell carcinoma (ESCC) and treatment with 5-Aza-cytidine, a demethylating agent, resulted in *WNT5A* reexpression and demethylation of its promoter in silenced cell lines (Li *et al.*, 2010). Also, ectopic expression of *WNT5A* inhibited proliferation and motility of the ESCC cells. Ying *et al.* (2008) studied *WNT5A* methylation and determined that the promoter region is hypermethylated in colorectal cancer and demethylation of this region resulted in reactivation of *WNT5A* expression. Similarly, hypermethylation of the *WNT5A* gene was detected in patient tissues with colorectal cancer, whereas no methylation was detected in the normal tissues from the same patients (Hibi *et al.* 2009).

Decrease in *WNT5A* mRNA levels was associated with *WNT5A* hypermethylation in acute lymphoblastic leukemia patient derived cell lines. *WNT5A* expression was restored after being treated with 5-Aza-cytidine (Roman-Gomez *et al.*, 2007). In another study, the methylation status of *WNT5A* promoter- exon 1 was analyzed and it was observed that there is hypermethylation of the promoter region in acute myeloid leukemia (Martin *et al.*, 2009). Conversely, in a study by Wang *et al.* (2007), three CpG sites in the

5' untranslated region (UTR) of *WNT5A* were consistently methylated in normal prostate cells but were hypomethylated in prostate cancer cell lines. These studies support the conclusion that altered DNA methylation of the *WNT5A* promoter genomic region is associated with *WNT5A* misregulation in cancer. Currently, nothing is published on *WNT5A* promoter B associated CpG island methylation.

#### *WNT5A Promoter A and B Regulation*

Previous research in this lab focused in transcriptional regulation from the distinct *WNT5A* alternative promoters A and B. Results from these studies formed the basis of this project. The individual promoter regions A and B were cloned into luciferase transfer vectors and deletion constructs were generated. These were used to analyze expression in different cell types and to identify specific gene regulatory regions. Results showed that promoters A and B were both active in Caco-2 (epithelial) and NIH 3T3 (fibroblast) cells and that 300-450 bp of upstream sequence are sufficient for maximal expression (Katula *et. al.* 2012). Significantly, promoter B constructs displayed distinct patterns of expression in two cell types and were more responsive to tumor necrosis factor (TNF)-alpha (Katula *et. al.* 2012). These results indicated that *WNT5A* promoters A and B are differentially regulated.

Taqman primer/ probes were designed to detect promoter A and B transcripts by qRT-PCR (Hsu 2011, Katula *et. al.* 2012). These primer/ probes were initially used to determine if endogenous promoter A and promoter B transcript levels varied during

differentiation and cancer progression. Promoter A and B transcript levels were quantified during differentiation of mouse fibroblast cells 3T3-L1 into adipocytes. Results showed that the ratio of A to B varied and promoter B transcript levels were the highest in undifferentiated 3T3-L1 cells. B transcripts decreased in adipocytes (Hsu 2011).

Promoter A and B transcripts were quantified in normal human osteoblasts and osteosarcoma cell line SaOS-2. Promoter B transcripts were nearly undetectable in the SaOS-2 cells. In normal osteoblasts, B transcripts were nearly 10 fold higher than A transcripts and approximately 450 fold higher than B transcripts in osteosarcoma cells. Promoter A transcripts were detected in both osteoblast and osteosarcoma cells but there were approximately 3.5X promoter B than A transcripts in the osteosarcoma cells. These data indicate that transcription from promoter B is greatly reduced during progression of osteoblasts to osteosarcoma. Promoter B reporter constructs were transfected into osteosarcoma SaOS-2 cells. All constructs were expressed at a relatively high level (Hsu 2011). This result indicates that the decrease in promoter B transcript levels in osteosarcoma is not due to altered transcription factors. The other likely possibility is that promoter B associated CpG islands become methylated, reducing transcription of promoter B.

## CHAPTER II

### MATERIALS AND METHODS

#### *Bioinformatics Search*

The NCBI epigenomics search tool (<http://www.ncbi.nlm.nih.gov/epigenomics>) was used to find *WNT5A* relevant data. This site contains experimental data from various epigenetic studies on many human cell types. I focused on the studies of DNA methylation data that were available for mesenchymal cell types including mesenchymal stem cells, chondrocytes, fibroblasts, and osteoblasts in the *WNT5A* genomic region. I focused on the DNA methylation status of the 6076 bp of intron 1, which includes promoter B, exon 1B and upstream sequences in the *WNT5A* gene (Fig. 2). I also looked at the CpG islands that were documented in these experiments. I compared the DNA methylation status of the mesenchymal stem cells, chondrocytes, and fibroblasts with each other and in relation to our identified CpG islands.

The database was also searched for the DNA methylation data of different samples of colorectal cancer and the DNA methylation status of the *WNT5A* intron 1 region compared. The DNA methylation status in relation to the identified CpG islands and the methylation status of each colorectal cancer to its normal colon mucosa cells, with respect to the CpG islands were compared. Some of the more relevant cell types were not available, e.g. osteoblasts

### *Identification of CpG Islands*

The CpG islands were identified using an online program called “CpG Embossplot”. The program searched the 6076 bp long WNT5A intron 1 sequence for putative CpG islands. The parameters for the search were that the regions should be more than 200 base pairs long. The GC percentage should be greater than 50% and an observed-to-expected CpG ratio greater than 60%.

### *Primer Design*

The primers were designed using an online program called “Methprimer”. The sequence of each CpG island along with 100-200 bp upstream and downstream sequences was entered into the program. Then the program used product size, melting temperature and primer size as parameters to generate the primers.

### *Cell Culture*

SaOS-2 osteosarcoma cells were obtained from the American Type Culture Collection (ATCC, CAT # HTB-85). The cells were grown in McCoy’s 5a Modified medium containing 15% fetal bovine serum and penicillin/streptomycin (50 I.U./ 50 µg per ml). The cells were grown at 37°C, 5% CO<sub>2</sub> humidified cell culture incubator.

### *Isolation of Genomic DNA*

SaOS-2 cells were plated at  $1 \times 10^5$  cell in 100mm plates and grown for 2-3 days. The cells were collected by trypsinization. The cells were pelleted by centrifugation (5 mins at setting #3 on a clinical centrifuge) and resuspended in 1X Phosphate Buffered Saline (PBS). The cells were counted using a hemocytometer and aliquots representing  $1 \times 10^6$  cells were transferred to a microfuge tube and pelleted by centrifugation. Genomic DNA was isolated from the cell pellets using a Genomic DNA Isolation Kit (Zymo research, D3004). The cells were lysed using 200  $\mu$ l of Genomic Lysis Buffer, then 10  $\mu$ l ZymoBeads was added to this and incubated at room temperature for 5 minutes. The mixture was centrifuged and the pellet was collected and washed twice with 200  $\mu$ l of DNA Wash Buffer. The DNA was eluted using 35  $\mu$ l Elution Buffer. The DNA concentration and purity was determined by reading the optical density at 260 and 280 nm. Osteoblast genomic DNA was purchased (ScienCell, #4609).

### *DNA Bisulfite Conversion*

The genomic DNA was bisulfite converted using the EZ DNA Methylation Kit (Zymo research, D5001). 500-550  $\mu$ g of genomic DNA was mixed with 5  $\mu$ l of M-Dilution Buffer and water in a 50  $\mu$ l reaction and incubated at 37°C for 15 minutes and 100  $\mu$ l C-T Conversion Reagent was added. The mix was incubated at 50°C for 12-16 hours then put on ice for 10 minutes. The reaction was mixed with 400  $\mu$ l of M-Binding Buffer and spun through a Zymo-Spin™ IC Column. The column was washed with 100

$\mu\text{l}$  of M-Wash Buffer and 200  $\mu\text{l}$  of Desulphonation Buffer was added to remove the bisulfite from the reaction. The converted DNA was eluted using 10  $\mu\text{l}$  of Elution Buffer. The final DNA concentration was determined by reading its optical density at 260 and 280 nm.

### *PCR Amplification and Subcloning*

Bisulfite specific primer sets for PCR amplification of CpG island regions from converted DNA were designed using an online tool called Methprimer (<http://www.urogene.org/methprimer/>). These primers are listed in Table 2. The sequence of each CpG island along with 100-200 bp of upstream and downstream sequence was entered into the program. The program then uses primer size and primer melting temperature to generate forward and reverse primers. These primers are complimentary to sequences flanking the CpG islands but lack any CpG's.

1-2  $\mu\text{l}$  of the bisulfite converted DNA with concentration 15- 30 ng/ $\mu\text{l}$  was typically used for PCR amplification using the generated bisulfite specific primers. The amplification was done using ZymoTaq DNA polymerase (Zymoresearch, E2001) and the amplification condition was determined using melting temperatures of respective primers for each region. The PCR product was verified by running 10 $\mu\text{l}$  of the products on a 2% agarose gel along with a 100 bp DNA marker and visualized with UV transillumination.

The verified PCR product (40  $\mu$ l) was run on a 1.5% low melt agarose gel. The bands at the correct sizes were excised and purified using the QIAquick Gel Extraction Kit (Qiagen, 28704). The gel piece was mixed with 3X gel volume of QG buffer and incubated at 50°C for 10 minutes. Gel volume of Isopropanol was added to the mix and spun in QIAquick Spin Columns. The column was washed using 750  $\mu$ l PE Buffer and eluted using 30 $\mu$ l Elution Buffer. The concentration of the purified PCR product was determined by reading the optical density at 260 and 280 nm. Then, purified PCR product 2-4  $\mu$ l with concentration 10-50 ng/ $\mu$ l was cloned into TOPO vector using the TOPO cloning kit (Invitrogen, 450641) and incubated at room temperature for 10 minutes. 2  $\mu$ l of the “vector – PCR mix’ was transformed into competent DH5 $\alpha$  *E. coli* (50  $\mu$ l). The mixture plus cells were incubated on ice for 10-20 mins and then heat shocked at 42°C for 30 seconds and placed on ice for 5 mins. The transformed *E. coli* was shaken horizontally for 1 hour at 37°C. 250  $\mu$ l S.O.C growth medium (Invitrogen,15544-034) was added to the cells. 150  $\mu$ l of the transformation growth media mix was plated on Luria Broth (LB) -Ampicillin (AMP) plates coated with 40  $\mu$ l of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) (40 mg/ml) and grown overnight at 37°C. The white colonies were picked and plated on a LB-AMP masterplate and grown overnight in LB-broth with 100  $\mu$ g/ml ampicillin at 37°C.

### *Plasmid Screening, Purification and Sequencing*

The plasmids in the minipreps of white colonies were screened for inserts. The individual bacterial colonies grown overnight in LB-AMP broth were pelleted by centrifugation. The supernatant was removed and the cells were resuspended and lysed using 200  $\mu$ l of Lysis Buffer (Lysozyme [Sigma-Aldrich, L7651], 0.15 mg, 8% Sucrose, 0.5 % Triton X-100, 50mM EDTA, 10 mM Tris-HCl, pH 8.0). The mix was boiled for 40 seconds, placed on ice for 10 minutes and centrifuged for 6 minutes at top speed. 10  $\mu$ l of the supernatant mixed with sample buffer was run on a 1.5% Agarose gel with a Lambda/Hind III marker. The gel was visualized with UV illumination and plasmids with inserts were picked.

Plasmids with verified inserts were grown overnight at 37°C and was purified from bacterial clones using the Promega Plasmid Miniprep Kit (Promega, A1460). The bacterial cells were pelleted by centrifugation and the supernatant was aspirated. The cells were resuspended in 200  $\mu$ l Resuspension Buffer and 200  $\mu$ l Cell Lysis Buffer was used to lyse the cells. 200  $\mu$ l of Neutralization Buffer was added and the mix was centrifuged for 6 mins. The supernatant was collected and mixed with Wizard Miniprep DNA Purification Resin and run through a Wizard Miniprep Column. The column was washed with 2 mL Column Wash Buffer and dried. The plasmid was eluted using 30-50  $\mu$ l of nuclease free water. The insert size was verified by using 500-550 ng of the plasmid and conducting a restriction digest using EcoRI (Promega, R6011). The restriction digestion reaction was run on a 1.5% Agarose gel and visualized using UV

transillumination and inserts were verified by band size. Verified plasmids were sent to Eurofin MWG Operon facility for sequencing.

#### *DNA Sequence Analysis*

Sequencing results were available from Eurofin. Each sequence was provided in different formats. Using MacVector format, the sequence was compared to a generated bisulfite converted sequence of that same region, which was assumed to be methylated. Hence, all the C's in the CpG's remained a C. all other C's were converted to T's. The obtained sequence was aligned to the bisulfite converted sequence using the MacVector alignment tool. Each CpG was visually examined. A methylated CpG in the experimental sequence would be aligned to the bisulfite converted CpG. An unmethylated CpG would be converted to a T and not aligned to the converted sequence

#### *5-Aza-cytidine Treatment of Cells*

5-Aza-cytidine was made by adding 200  $\mu$ l of 1:1 solution of Glacial Acetic Acid and Nanopure water into the 100 mg 5-Aza-cytidine stock. 10  $\mu$ l of the 5-Aza-cytidine stock solution was added to 90  $\mu$ l of 1:1 of Glacial Acetic Acid and Nanopure water solution to make a 1:10 dilution.

The SaOS-2 cells were plated in 100 mm plates at a density of per 100 mm plates and grown overnight. The cells were treated with 0, 1, 5 and 10  $\mu$ M 5-Aza-cytidine (InvivoGen, met-adc-1) on day 1 and 3. The 1, 5 and 10  $\mu$ M 5-Aza-cytidine final

concentrations were made according to Table 3. The On day 4, the cells were collected and stored at -80°C. RNA and DNA was isolated from the cells.

Table 1. Preparation of 1 , 5 and 10  $\mu$ M 5-Aza-cytidine final concentrations

Final 5-Aza-cytidine concentration	5-Aza-cytidine 1:10 dilution	1:1 Glacial Acetic Acid and Nanopure water
0 $\mu$ M	0 $\mu$ l	5 $\mu$ l
1 $\mu$ M	0.45 $\mu$ l	4.05 $\mu$ l
5 $\mu$ M	2.25 $\mu$ l	2.25 $\mu$ l
10 $\mu$ M	4.5 $\mu$ l	0 $\mu$ l

*RNA Isolation, cDNA Preparation and qRT-PCR*

First, the medium was removed from the cells and the cells were washed with cold, sterile 1X PBS. 5 mL 1xPBS was added and the cells were scrapped from plate and transferred to a 15 mL Faclon Tube. The cells were centrifuged for 5 minutes at setting #4 in a clinical centrifuge. The PBS was removed and the cells were quickly resuspended in 175 $\mu$ l RNA Lysis Buffer. RNA was isolated using the Promega SV Total RNA Isolation Kit (Promega, Z3100). 350  $\mu$ l of RNA Dilution Buffer was added and mixed. The sample was heated at 70°C for 3 minutes and the cleared lysate was removed to a clean tube and 200 $\mu$ l of 95% ethanol was added. The mixture was spun through a RNA Isolation Column for 1 minute in a microfuge. The column was washed with 600 $\mu$ l of RNA Wash Solution and incubated for 15 mins at room temperature. 50ul of DNase mix was added to the top of the resin and was incubated for 15-20 mins at 20-25°C and the reaction was stopped with 200 $\mu$ l of DNase stop solution. The columns were washed again with RNA Wash Solution and the RNA was eluted with 100 $\mu$ l of Nuclease free

water. The quantity and the purity of the RNA was determined by reading at 260 and 280 nm.

The isolated RNA was converted into cDNA using the Maxima First Strand cDNA Synthesis Kit (Maxima, K1641). The 1-5  $\mu\text{g}$  of RNA was used to make a 20  $\mu\text{l}$  master mix containing 1x Reaction Mix, Maxima Enzyme Mix and Nuclease Free Water. The reaction mix was incubated at 25°C for 10 minutes and 50°C for 15 minutes. The reaction was terminated by incubating the mix at 85°C for 5 minutes. The resulting cDNA strand was used to perform a qRT-PCR reaction to quantify promoter A and promoter B specific transcripts. A total assay set required 48 samples. This included three replicas of each target transcript (promoter A, promoter B and GAPDH as an internal control) for each sample cDNA (0, 1, 5, 10  $\mu\text{M}$  5-Aza-cytidine). In addition, promoter A and B PCR standard curves were included for quantification of transcript numbers.

## CHAPTER III

### RESULTS

#### *Identification of CpG Islands*

The *WNT5A* promoter B start of transcription and exon 1B are located in intron 1 of the promoter A transcription unit (see Fig. 2). As such, we wanted to identify CpG islands within the intron 1 sequences. These sites are likely to influence promoter B transcription. Initially, intron 1 was analyzed using “Emboss CpG plot” (see Materials and Methods). Five CpG island regions were identified. Then, the CpG islands included in these same sequences that were identified by the University of California Santa Cruz (UCSC) Genome Browser were considered giving a total of six islands. Table 2 includes the base pair size and number of CpG’s for each region. The DNA sequence and location of each CpG for each region is shown in Figure 3.

Table 2. *WNT5A* Intron 1 CpG Island Characteristics

Region	Size (base pairs)	Number of CpG’s
Region-1	343	27
Region -2	617	39
Region -3	441	24
Region -4	350	27
Region -5	578	25
Region -6	385	26

A.

```
REGION 1
          1      2 3      4      5      6
CAGGATAATGATTCAATTCAACAAATATTTACCAAGCGCCACTGCGCGGGCACCGGGA CGGTGTGTGGC CGGGGCT
7
CGGGATGGGAAAGGGACTCCTTGGTTGAGAGAACC CGCAGAGGA ACTAGGGGCCAACTACTCCAGAGCGGCGTACA
11      12      13 14      15      16 17
CCTTCCC CGACACCCCC CGCAC CGA CGGAGGCCAGATTGTAGGCTTGCCTTGGC CGAGTAACT CGAGCGCTTCAGG
18      19 20      21 22 23      24      25 26
CCCCTTTGCCTTTAAAGAGTGCCTGGCGGGGGCCAGCGCGCACCC CGGCGCGGGGAGCGGGGT CGCGTTATTAGCAT
27
TATTAGC CGCCACTGGGTGAGCTCAGGCAC TTTGC
```

B.

```
REGION 2
          1      2      3      4      5 6      7
TAGTTTCTAGACAGACTCTGCCAGGGGAGTTT CGAGGGGGCAATG CGGAGAGTG CGCAGCGAAC CGCGGGC CGACAA
8      9      10      11
ATGTGTGTGTGTC CGAGTGTGTGGATCCTTGGCTG CGTGTGTGTG CGTGTGTGTTGTGTGTGTAGGGGAGTTGTTC
12      13 14      15
TCGGTCCAGGACG TAGTAGCCTAGAGTCCCCTGTCTTCTCCTCCCCTCCCC CGTGCCTCCGCTGCTCAAGGGGTT
16 17      18      19 20      21 22 23      24
AGCACTATG CGCGCAGAACT CGGAGCCATCCC CGCGGCTTTTCTTCCC CGCGGTGCGGTGC CGGCTGCCCCCTGTTC
25      26      27      28 29 30
GGC CGCCTTGAGGTAGACTCCTG CGAGCCT CGGTTCTTGGGG CGGGCGGG CGGCAGCTGCTCCCCTCTCCCTCC
31      32
CACCCCTGCTCCCTTCATCCCTCC CGACCACAGACCCCACTCTCCAGCAGGGGCCCT CGGAGGCCCCAGAGGAC
33      34 35 36 37      38      39
CAGTTT CGCTGCGCGCGCGCTGCAATA CGGAAGGGGCCA CGGGTCCAGCCAGCAAA TGGGACTCGGCCAGCT
```

C.

```
REGION 3
          1      2
GGGAGAGGATCTTTGCTTGGCCTTTTATCTATTCTCTTAAGGGACTGCTG CGGAGGAGGAGTTGGGTGTTG CGAGA
3      4 5      6 7
CTTTAGGTGTGCATC CGGCTGCTCCC CGCGCCCTCCGAA CGTCTCTGAACAATTTTGCTACTGAGGTTTCTCTCT
8      9      10      11 12 13
CCCTCATCCCTCATCA CGCCTGGCC CGCAC CGTGGTTGGTAAAGAAGGATCAAGAACACCTTTAATC CGACGCGAAG
14 15      16 17      18
GCTTTGAGGGATACATAGAAAAGGGAGCCA CGT CGTTGAGCTGCTCTGGATTGAAGAGGAGGA CGCGGGAA CGCCCTC
19      20      21      22 23
CTCCACCTCACTCCTCTGGTG CGCTGAGCCCTCAAA CGCCCCAGTCTCCTCCC CGTGGCA CGCGAGGAAGATTC
24
TCAGTGTCTTACAGAGTCATCTTCCCCTGAAGATCTCAGTGTCTTACAGAGTCATCTTCCCCTGAGCCC CGAAA
GTGTTGAAAAACATTTAGCCCC
```

D.

```
REGION 4
          1           2
CCTGGAGCCAGGGAGACCCAAGTCTTGGTGACCGGATGGGGCCCGCTCTCAGTTGGCCTGGGCTCTGGGAACTGGTGG
          3           4           5           6
ACTCTCCCTGGGGGCTTCGGGCTGGGAGTGGGTTCGGTTTGTGTGGCTTCGGCTCTAACAAAGAGATCCGCTGAATC
          7 8           9           10 11 12 13 14 15
CGCCGAATCTGTTATCAATTTCTCTGCTGCTTGAGCCCAGCCCCAGCGCCCGCTCGCCGCGAAGCTTGAAAAGTG
          16 17           18 19           20 21           22 23
CACGCGGCCAGCACCAATCTGGGCCTGACTCGGAAACATGTCGACGCTGTGTGTCTATGCAACGCGTGTGAGTGT
          24           25 26 27
GTAAATGTGCAAGAGTGTGAATGTGTATGATGTGTGTGCAACGCGGCATCGGCTGCCCTTGGGGAGAGTTGACTTTGC
AGCCTGGGCTG
```

E.

```
REGION 5
          1           2
AGGGCTGCCTAGGTCCCCTCTGCCCTTTTACGGCACAGGTTCCAAGCCAGGCTCTTCCACCGCCTTAAAGAGGCTC
          3           4           5
ACCTTCTCTTTCTTTCTGTGGAAGGGGCTCCTCAGGGGCTATGGGCGATGCAGTGGCGCAGGTTAGACTTACGT
          6           7           8           9           10 11 12
GTAAGGGGATTTTTAAAACCGCTCCTCCACCAGCACCAGCCACCTACTCGCTCCGCGCGCCTACAGGTGGAGA
          13 14
AGTCACCAGTGGGGAGGAAAGCGCAGCGGAAGCTTCCAAGGCCAACTCCTACCCCTGAAATTTCTCAGGAAGGGAACC
          15 16           17 18           19 20           21           22
TTCCGCGCTGGGGGCTCTTTGCCCTGGAATCGATGCGCCAGCTGCGGCTCGGAAGCCAGCGCCTCTGGCCCCTC
          23 24           25
TGGACTCATCTGCAAGGGCTCTGGCTCGCCCGCACCCCCACCTTTGGCACTGAC
```

F.

```
RRGION 6
          1           2
CCCTCCCTAGTCTGGCTGAAGAAAGTCTTAAGGAAATCCGGGCTGCTCTTCCCCTGCTGGAAGTGGCTTTCCCAC
          3 4           5           6           7 8 9
ATCGGCTCGTAAACTGATTATGAAACATAAGATGTTAATTCCGAGCTGCATTCCCAGCTGGGCACTCTCGCGGCT
          10 11           12 13           14 15
GGTCCCAGGGGCTCGCCCCCACCCTGCCCCTTCCCTCCCGGCTCCTGCCCCATCCTCCACCCCCCGCGCTGGC
          16           17 18 19           20 21           22
CACCCCGCCTCCTTGGCAGCCTCTGGCGCAGCGCGCTCCACTCGCCTCCGTGCTCCTCTCGCCATGGAATTAATT
          23           24           25 26
CTGGCTCCACTTGTGTGCTCGGCCAGGTTGGGGAGAGGACGGAGGTGGCCGACGCGGTTCTGAGTGAATTACCC
```

Figure 3. A-F, Sequence of *WNT5A* intron 1 CpG islands. Each CpG is highlighted in red and numbered.

These CpG islands were the focus of the DNA methylation analysis in this study. More recently, our CpG islands were compared to the UCSC Genome Browser CpG island map and the NCBI for the *WNT5A* intron 1 (Fig. 4).

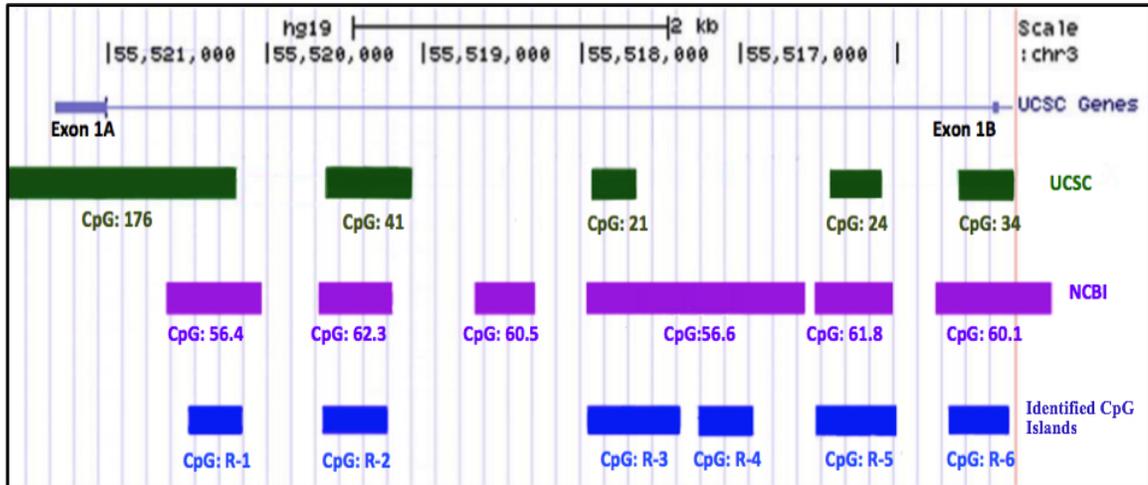


Figure 4. Comparative location and size of the selected CpG islands. CpG islands found in this proposal are compared with the UCSC Genome Browser CpG island map and the NCBI Epigenomic Browser CpG Island map.

### *Bioinformatics Analysis of the WNT5A Intron 1 Sequences*

Numerous genomic scale epigenetic analyses have been completed and the resulting DNA methylation data deposited into searchable databases. The NCBI epigenome database was searched to generate an “epigenetic portrait” of the *WNT5A* intron 1 region. I chose to look at data that were related to *WNT5A* normal expression and some cancers having misregulated *WNT5A* or *WNT* signalling. *WNT5A* is primarily expressed in mesenchymal cells, hence experiments including chondrocytes, fibroblasts, adipocytes, osteoblasts and mesenchymal stem cells were searched.

DNA methylation data from two different donor samples of mesenchymal stem cells derived from the bone marrow showed that there is very little methylation in each of the six CpG islands. Similarly, two different human fibroblast cell lines (Fib 20 and Fib 17), derived from the H9 human embryonic stem cell line had little methylation in the six intron 1 CpG islands (Fig. 5). And, data from three different donor samples of chondrocytes derived from the bone marrow also showed very little methylation (Fig. 5). These results suggest that the *WNT5A* intron 1 region is not methylated or has very little methylation in cells in which *WNT5A* is normally expressed. Unfortunately, DNA methylation data was not available for all cell types of interest. For example, while epigenetic experiments were completed with osteoblasts there were no DNA methylation data. There were no DNA methylation data for osteosarcoma cells.

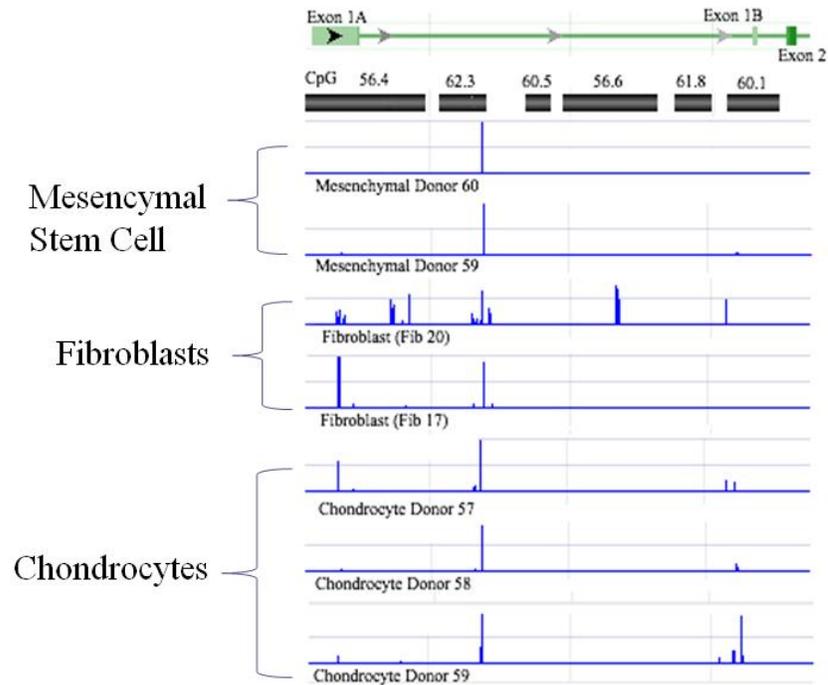


Figure 5. *WNT5A* intron 1 DNA methylation profiles. Methylation profiles from different samples of Fibroblast, Chondrocytes and Mesenchymal Stem Cells. The *WNT5A* intron region is shown above. Vertical lines indicate that some DNA methylation was detected at that position.

*WNT5A* expression is also associated with colorectal cancer (King *et al.* 2008, Hibi *et al.* 2009, Rawson *et al.* 2011) The NCBI epigenomic database contains DNA methylation data from five different colon cancer samples with their respective normal cells from the colon mucosa (Fig. 6). The colon cancer sample from a 31 year old patient shows a higher level of methylation in the CpG island Regions 3 and 4 when compared to methylation data from its normal colon mucosa tissue. Another sample from an 81 year old colon cancer patient showed an increase in the level of methylation in all six CpG islands. Other colon cancer samples from patients aged 67, 70 and 85 did not show much

difference in methylation between the cancer and corresponding normal tissue. When compared to the DNA methylation data for human mammary epithelial cells, normal colon mucosa cells had a higher level of methylation in CpG island Regions 3 and 4 (data not shown). These data suggest that in normal colon epithelial cells, CpG island Regions 3 and 4 are more susceptible to DNA methylation. In transformed colon epithelial cells, the amount and regions of DNA methylation generally increases.

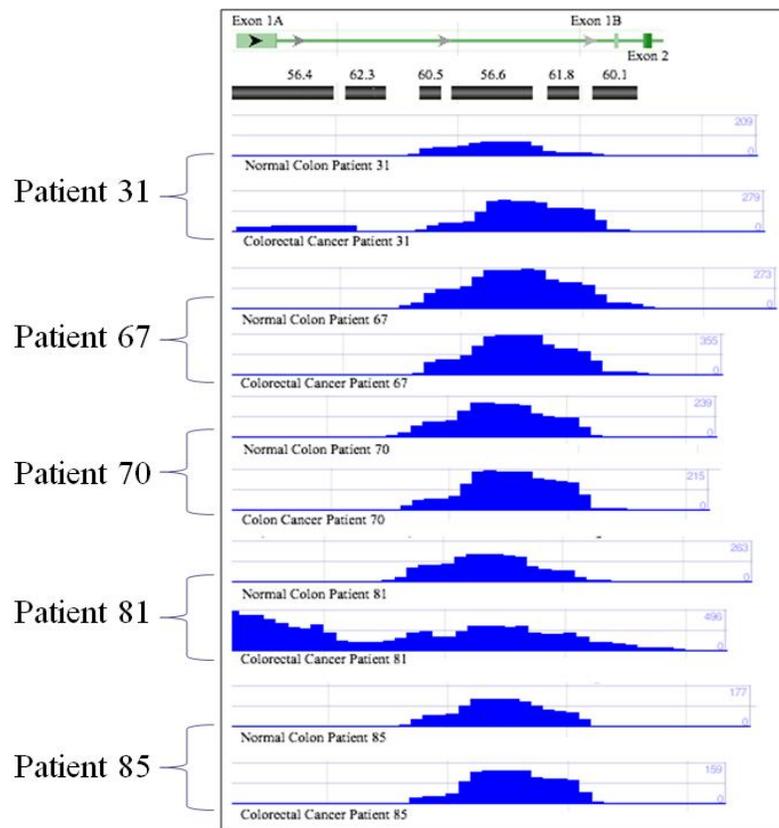


Figure 6. *WNT5A* intron 1 DNA methylation profiles from different Colorectal Cancer patients compared to Normal Colon in the patients. The *WNT5A* intron 1 region is shown above. The black areas indicate relative levels of DNA methylation at that particular sequence. Note that the scales vary.

*DNA Methylation Analysis of WNT5A Intron 1 CpG Islands*

The methylation status of the six CpG islands was analyzed by bisulfite sequencing. The sodium bisulfite specific primers designed to amplify each CpG island with their respective amplification condition are shown in the Table 3.

Table 3. Bisulfite Sequencing PCR Primers

Primer Name	Type	Sequence (5'-3')	Amplification Condition	Size Region
W5AR1F	Forward	AGTAATAGGATAATGATTTAATTTAATAAA	95°C- 10min /95°C - 30s/45°C - 30s/ 63°C - 30s	393
W5AR1R	Reverse	CAAATACCTAAACTCACCAATA		
W5AR2F	Forward	AGTTTTTAGATAGATTTTGTAGGGGAGT	95°C- 10min /95°C - 30s/45°C - 30s/ 63°C - 30s	255
W5AR2R	Reverse	CATAATACTAACCCCTAACACCC		
W5APrBR2HF	Forward	GTTTAAGAGTTTTTGTTTTTTTT		335
W5APrBR2HR	Reverse	AAATACCAACCCTATATCCAATAAC		
W5aR3F#2	Forward	GAGGATTTTTGTTGGTTTTTTATT	95°C- 10min /95°C - 30s/55°C - 30s/ 63°C - 30s	441
W5AR3R	Reverse	AAACTAAATTTTTCCAACACTTTC		
W5AR4F	Forward	GTTTTGGGAATTGGTGGATT	95°C- 10min /95°C - 30s/45°C - 30s/ 63°C - 30s	329
W5AR4R	Reverse	AACCCAACTACAAAATCAACTCTC		
W5AR4F3H2	Forward	GAATTTGTTATTAATTTTTTGTGTTTG	95°C- 10min /95°C - 30s/58°C - 30s/ 63°C - 30s	226
W5AR4R3H2	Reverse	TACAAAATCAACTCTCCCAAAA		
W5APrBR5nF	Forward	AGGGTTGTTTAGGTTTTTTTGT	95°C- 10min /95°C - 30s/45°C - 30s/ 63°C - 30s	578
W5APrBR5nR	Reverse	AACTCCAATTCCTTAATCCTAAT		
W5AR6F	Forward	TTTTTTTAGTTGGTTGAAGAA	95°C- 10min /95°C - 30s/55°C - 30s/ 63°C - 30s	393
W5AR6R	Reverse	CCTAAATAATCACTCAAAAACCC		
W5AR6H2F5	Forward	AATTAATTTGGTTTTATTTGTTGTT	95°C- 10min /95°C - 30s/55°C - 30s/ 63°C - 30s	96
W5AR6H2R5	Reverse	TCAATCCCTCTAAATAATCACTC		

The primers were used to amplify their respective sodium bisulfite converted regions for human osteoblast and osteosarcoma SaOS-2 DNA. The amplified regions

were verified by size and gel purified. The purified PCR products were cloned into a TOPO vector and transformed into DH5 $\alpha$  *E. coli*. The colonies with plasmid inserts of correct size were purified and sent to Eurofins MWG Operon for sequencing. The number of independent PCR colonies analyzed for each CpG island region is listed in table 4. The sequence results obtained for *WNT5A* Region 1 shows that all 27 CpG's present in the region are unmethylated for both osteoblast and osteosarcoma SaOS-2 DNA (Fig 7A). The sequence data obtained for *WNT5A* Region 2 shows that all 39 CpG's present in the region are not methylated for both osteoblast and osteosarcoma SaOS-2 DNA (Fig 7B). The CpG's are highlighted and numbered according to Figure 3.

Table 4. Number of independent PCR colonies analyzed for each CpG island region.

<b>Regions</b>	<b>No. Of Clones analyzed (Osteosarcoma)</b>	<b>No. Of Clones analyzed (Osteoblast)</b>
<b>CpG Island Region 1</b>	<b>3</b>	<b>2</b>
<b>CpG Island Region 2</b>	<b>1</b>	<b>1</b>
<b>CpG Island Region 2H</b>	<b>3</b>	<b>1</b>
<b>CpG Island Region 3</b>	<b>3</b>	<b>3</b>
<b>CpG Island Region 4</b>	<b>4</b>	<b>2</b>
<b>CpG Island Region 5</b>	<b>4</b>	<b>1</b>
<b>CpG Island Region 6</b>	<b>1</b>	<b>3</b>

A.

CpG Island Region 1

```
TTAGTAATAGGATAATGATTTAATTTAATAAATATTTATTAAGCGTTTATTGCGCGGGTATCGGGACGGT
|||||
TTAGTAATAGGATAATGATTTAATTTAATAAATATTTATTAAGTGTATTATTGCGTGGGTATCGGGATCGT
|||||
1 2 3 4 5

GTGTGGTTCGGGGTTCGGGATGGGAAAGGGATTTTTGGTTGAGAGAATTCGTAGAGGAATTAGGGGTTA
|||||
GTGTGGTTCGGGGTTCGGGATGGGAAAGGGATTTTTGGTTGAGAGAATTCGTAGAGGAATTAGGGGTTA
6 7 8

ATTATTTTAGAGCGGCGTATATTTTTTCGATAGTTTTTCGTATCGACGGAGGTTAGATTGTAGGTTTTGT
|||||
ATTATTTTAGAGTGGTGTATATTTTTTCGATAGTTTTTCGTATCGATGGAGGTTAGATTGTAGGTTTTGT
9 10 11 12 13 14

TTTGGTCCAGTAATTCGAGCGTTTTAGGTTTTTTGTTTTAAGAGTGTTCGGCGGGGGTTAGCGCGTA
|||||
TTTGGTCCAGTAATTCGAGCGTTTTAGGTTTTTTGTTTTAAGAGTGTTCGGCGGGGGTTAGCGCGTA
15 16 17 18 19 20

TTTCGGCGCGGGAGCGGGTTCGGGTTATTAGTATTATTAGTCCGTTATTGGGTGAGTTAGGTATTTTGT
|||||
TTTGGTTCGGGAGTGGGTTGGTATTAGTATTATTAGTCCGTTATTGGGTGAGTTAGGTATTTTGA
21 22 23 24 25 26 27
```

B.

CpG Island 2

```
GATAGATTTTGTAGGGGAGTTTCGAGGGGTAATGCGGAGAGTCCGTAGCGAATCGCGGGTTCGATAAAT
|||||
GATAGATTTTGTAGGGGAGTTTCGAGGGGTAATGCGGAGAGTCCGTAGCGAATCGCGGGTTCGATAAAT
1 2 3 4 5 6 7

GTGTGTGTGTTCCGAGTGTGTGGATTTTTGGTTGCGTGTGTGCGTGTGTGTTGTGTGTAGGGGAGT
|||||
GTGTGTGTGTTCCGAGTGTGTGGATTTTTGGTTGCGTGTGTGCGTGTGTGTTGTGTGTAGGGGAGT
9 9 10

TTTAGGACGTAGTAGTTTTCGTTTTTTTTTATTTTTTCGATTTTTTAACCGAAGATTTTTTTAGGAGGTT
|||||
TTTAGGACGTAGTAGTTTTCGTTTTTTTTTATTTTTTCGATTTTTTAACCGAAGATTTTTTTAGGAGGTT
11 12 13 14

CGGTTTAAGAGTTTTTTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCGTCCGTTTCGGTGT
|||||
CGGTTTAAGAGTTTTTTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCGTCCGTTTCGGTGT
15 16 17 18

TTATGCGCGTAGAATTCGGAGTATTTTTCGCGGTTTTTTTTTTTTTTTTTTTTTTTTCGCGGTGCGGTGTCGGT
|||||
TTATGCGCGTAGAATTCGGAGTATTTTTCGCGGTTTTTTTTTTTTTTTTTTTTTTTTCGCGGTGCGGTGTCGGT
19 20 21 22 23 24 25 26 27

GTTGGTCCGTTTTGAGGTAGGATTTTTGCGAGTTTTCGGTTTTTTGGGGCGGGCGGGCGGGTAGTTGTTTT
|||||
GTTGGTCCGTTTTGAGGTAGGATTTTTGCGAGTTTTCGGTTTTTTGGGGCGGGCGGGCGGGTAGTTGTTTT
28 29 30 30 31 32

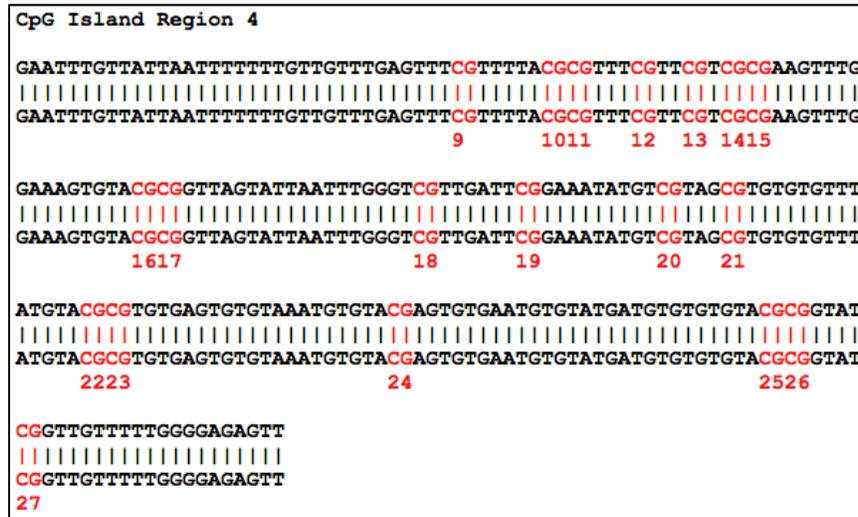
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCGATTATTAGATTTTATTTTTTTAGTAGGGGTTT
|||||
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCGATTATTAGATTTTATTTTTTTAGTAGGGGTTT
33

TCGGAGGTTTTTAGAGGATTAGTTTCGTTCCGTCGTCGGTCCGTTGTAATCGGAAGGGGTTAT
|||||
TCGGAGGTTTTTAGAGGATTAGTTTCGTTCCGTCGTCGGTCCGTTGTAATCGGAAGGGGTTAT
34 35 36 37 38 39
```

C.



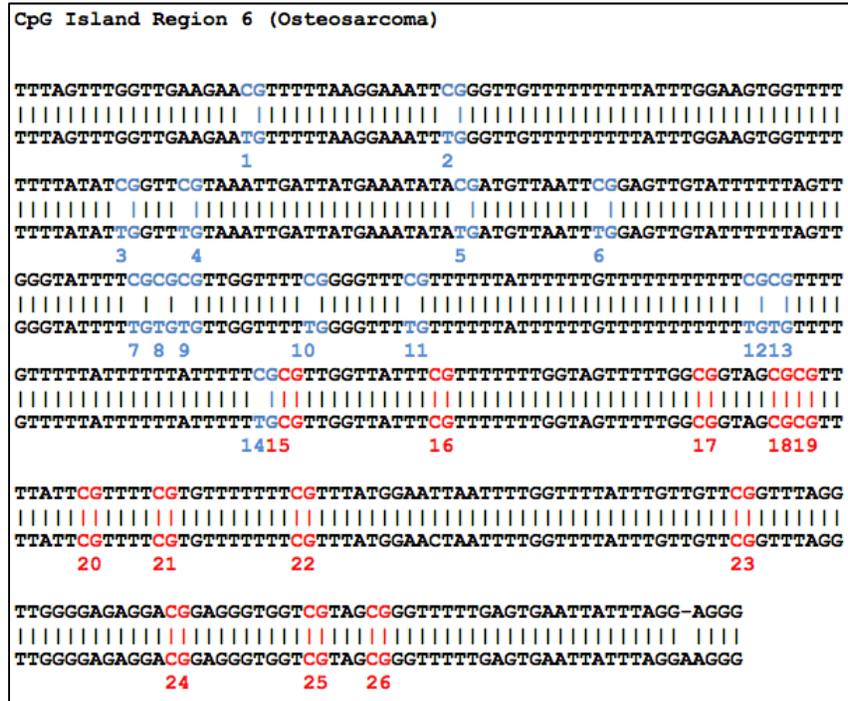
D.



E.



F.





The sequencing results for *WNT5A* region 5 shows that all 27 CpG's present in this region are not methylated in the osteoblast DNA but CpG's 9-27 are methylated in the osteosarcoma SaOS-2 DNA. Data were not available for CpG's 1-8. The results for CpG island region 6 are distinct. In osteoblasts all 26 CpG's in this region are unmethylated. However in the osteosarcoma SaOS-2 DNA CpG's 1 through 14 are unmethylated but CpG's 15 through 26 are methylated. The methylated CpG's of *WNT5A* region 6 include sequences just upstream of promoter B exon 1B and sequences within exon 1B. Presumably, the beginning of exon 1B is the promoter B start of transcription. The methylation status of each CpG in all six CpG regions for osteosarcoma and osteoblasts are illustrated in Figure 8.

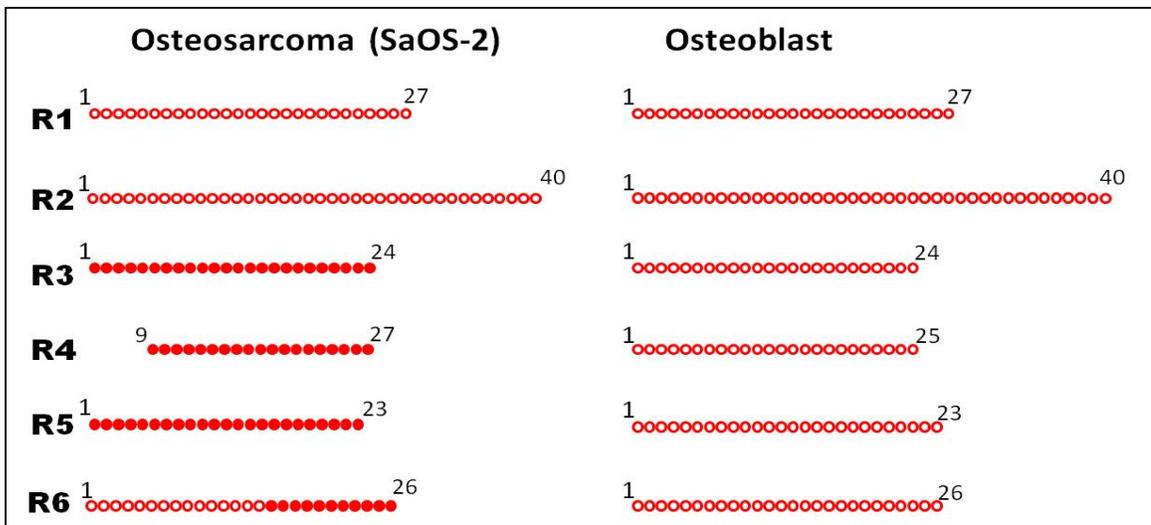


Figure 8. Methylation status of *WNT5A* intron 1 CpG islands. Regions (R) 1-6. Methylation status of *WNT5A* intron 1 CpG islands. Regions (R) 1-6. The closed circles represent methylation and the open circles represent no methylation. The numbers indicate the position of each CpG within the island.

### *Reactivation of Promoter B with 5-Aza-cytidine*

The data obtained previously in our lab showed that *WNT5A* promoter B transcripts are not expressed in osteosarcoma cells (SaOS-2). Sequence data from the DNA methylation analysis showed that CpG Regions 3, 4, 5 and a partial region of 6 are methylated and this methylation could be the reason for the decreased expression of *WNT5A* promoter B transcription. A demethylation experiment was done in order to test whether removal of these methyl groups would lead to an increase in *WNT5A* promoter B transcript levels. I treated osteosarcoma (SaOS-2) cells with different amounts of 5-Aza-cytidine, a demethylating agent for 4 days. The treated cells were collected and RNA was isolated and used to conduct a qRT-PCR analysis of total *WNT5A*, promoter A and promoter B transcripts.

The data obtained from the qRT-PCR analysis of total *WNT5A* and promoter A in SaOS-2 cells treated with 0, 1, 5 and 10  $\mu\text{M}$  of 5-Aza-cytidine show that in cells mock treated with 0  $\mu\text{M}$  5-Aza-cytidine there is no difference between the levels of the total *WNT5A* and promoter A transcript. Cells treated with 1  $\mu\text{M}$  of 5-Aza-cytidine show no significant change in the level of promoter A and total *WNT5A* transcripts. At 5 and 10  $\mu\text{M}$  concentrations, promoter A transcripts slightly decreased (Fig. 9, top).

The qRT-PCR analysis of promoter B transcripts in SaOS-2 cells treated with 0  $\mu\text{M}$  5-Aza-cytidine there is no promoter B transcripts in osteosarcoma cells. However, in cells treated with 1  $\mu\text{M}$  5-Aza-cytidine there is a 120 fold increase in transcript levels of promoter B compared to untreated cells. In cells treated with 5 and 10  $\mu\text{M}$  5-Aza-cytidine

the levels of transcript from promoter B increases 65 fold and 40 fold, respectively (Fig. 9, bottom).

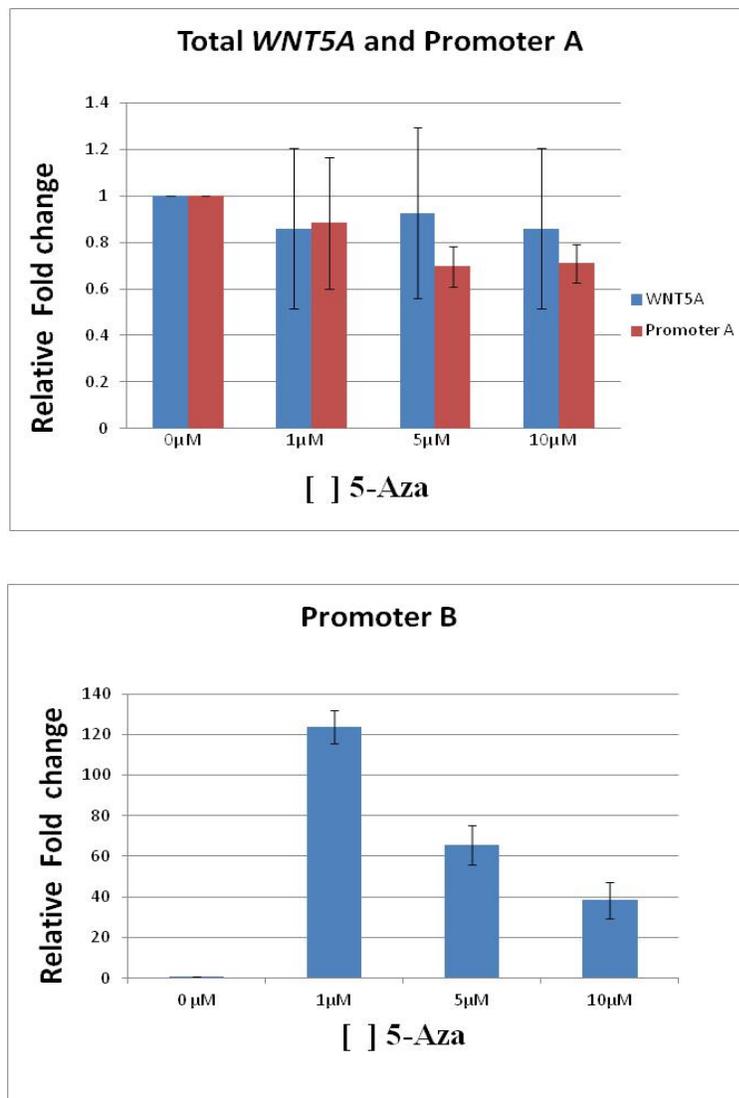


Figure 9. Reactivation of Promoter B after treatment with 5-Aza-cytidine. SaOS-2 cells were treated for 4 days with the indicated amounts of 5-Aza-cytidine. RNA was isolated and levels of total WNT5A, promoter A and promoter B specific transcripts were determined by qRT-PCR. Fold change is relative to untreated controls/ Error bars represent standard error. n=3

*DNA Methylation Analysis after Promoter B Reactivation of WNT5A Intron 1 CpG Islands*

I found that promoter B was reactivated by 5-Aza-cytidine. Next we wanted to determine which of the CpG islands are being most affected by the drug. It is likely that the regions having the most extensive demethylation have a role in regulating promoter B transcription. I performed bisulfite sequencing on CpG Regions 3, 4, 5 and partial region 6 of 1  $\mu$ M 5-Aza-cytidine treated SaOS-2 cells. Regions 1 and 2 were not analyzed since they were found to be unmethylated in osteosarcoma. The results indicate that demethylation is variable within the different regions, based on the analysis of multiple clones for each region and that Regions 3, 4 and 5 are more resistant to demethylation whereas, Region 6 shows greater demethylation. The methylation data were obtained for CpG regions 3, 4, 5 and a partial region of 6 for cells treated with 1  $\mu$ M 5-Aza-cytidine. The results show that different clones have different demethylation patterns (Fig. 10). The 5 clones obtained for CpG region 3 were named G5, G7, G9, Q6 and Q10. Clone G5 has CpG 4 demethylated. Clone G7, G9 and Q10 have CpG 24 demethylated. Clone Q6 has CpG's 5, 6 and 27 demethylated. The 5 clones obtained for CpG region 5 were named L10, L11, T6, T14, and T19. Clones L10 and T6 had demethylation in CpG 20 and clones T14 and T19 had demethylation in CpG 22. Clone L11 showed no demethylation.

The methylation analysis for region 6 was possible for only CpG 22-26. Among the three clones obtained for region 6 (R4, R11, and R16) clones R4 and R6 show CpG's

23-26 demethylated while clone R11 shows no demethylation. The one clone (S15) obtained for CpG island region 4 CpG 6-27 shows no demethylation. These results are illustrated in Figure 10. Additional clones are being analyzed.

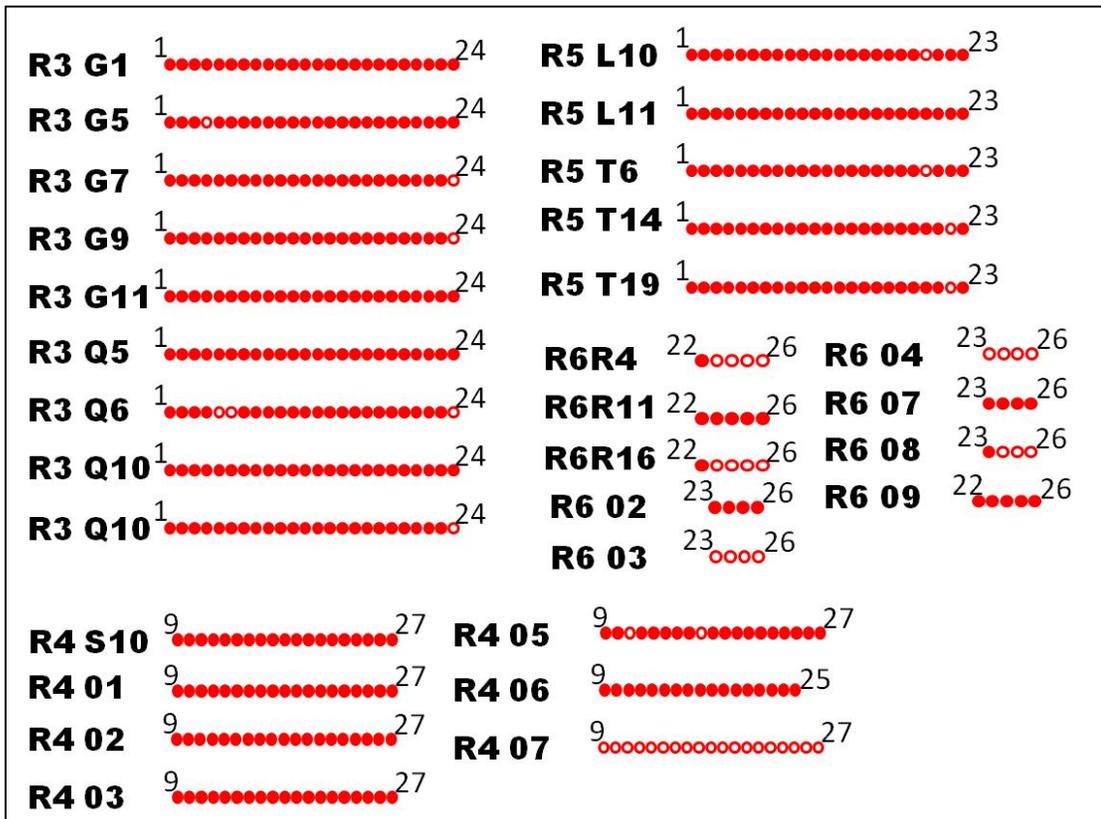


Figure 10. Methylation status of *WNT5A* intron 1 CpG islands in osteosarcoma cells treated with 1  $\mu$ M 5-Aza-cytidine. Regions (R) 3-6. The closed circles represent methylation and the open circles represent no methylation. The numbers indicate the position of each CpG within the island.

## CHAPTER IV

### DISCUSSION

#### *Summary of Project and Major Findings*

In this study, the DNA methylation status of six identified CpG islands associated with the *WNT5A* promoter B were analyzed in normal osteoblasts and the osteosarcoma cell line SaOS-2. My results showed that CpG island Regions 3 to 5 were completely methylated in the SaOS-2 DNA. Significantly, Region 6, which includes the promoter B start of transcription and exon 1B, was only partially methylated. Regions 1 and 2 were completely unmethylated. In contrast, all CpG island regions 1 to 6 were unmethylated in normal osteoblast cells. Treatment of SaOS-2 cells with 5-Aza-cytidine, a DNA demethylating agent, resulted in the reactivation of promoter B and increased promoter B transcripts. Bisulfite sequence analysis of the CpG islands of the 5-Aza-cytidine treated cells indicated that Region 6 is more prone to demethylation than the other CpG island regions. These results correspond to some of the bioinformatics data for the *WNT5A* region. In particular, in cells that normally express *WNT5A* (fibroblast, chondrocytes, and mesenchymal stem cells) there is little or no methylation of Regions 1 – 6. However, in some colorectal tumor tissue there is extensive methylation within intron 1. And, in adjacent normal colon mucosa tissue, Regions 3 and 4 showed some degree of methylation.

### *Six CpG Islands were Identified in the WNT5A Intron 1 Region*

The assumption was that sequences in intron 1 are relevant to promoter B expression. In order to identify these sequences we used an online computer program “EMBOSS CpG Plot” to screen the region for CpG islands. We identified 6 CpG islands and then we compared them to the CpG islands present in NCBI Epigenomics Browser and UCSC Genomic Browser. Figure 4 shows that our CpG islands are similar to the CpG islands present in the online databases. When our CpG islands are compared to the CpG islands in the UCSC Genome Browser, our Region 4 is not present in the browser. Our Region 1 contains only a part of UCSC’s CpG Region 176, which is likely to be associated with promoter A expression and extends across Exon 1A. Our Region 1 is included in *WNT5A* intron 1 and contains only the most 3’ region of UCSC CpG 176. However, the proximity of the Regions 1 and 2 to promoter A makes it more likely that methylation of these islands would impact promoter A function to a greater degree. In fact, methylation analysis of *WNT5A* promoter A have focused on CpG’s in exon 1A and of intron 1 (Ying *et. al.* 2008).

Region 6 most certainly is associated with promoter B expression. Moreover, using promoter B luciferase reporter constructs, 356 bp upstream of exon 1B was shown to be sufficient for maximal expression in SaOS-2 osteosarcoma cells (Hsu 2011). This result also supports the conclusion that Region 6 CpG is likely to have particular importance in regards to promoter B regulation. CpG island Regions 3, 4 and 5 are less likely to have an influence on either promoter A or B activity.

*Low Levels of DNA Methylation of WNT5A Intron 1 in Normal Mesenchymal Cells but Greater DNA Methylation in Colorectal Tumor*

Our bioinformatics data search showed that there is only a limited number of studies relevant to *WNT5A*. There were no DNA methylation data present for osteosarcoma cells and only histone methylation data for osteoblast cells. Some DNA methylation data were present for cells that express *WNT5A* such as mesenchymal stem cells, fibroblasts and chondrocytes, which showed that the CpG islands had no or very little methylation. These results suggest that in normal cells that express *WNT5A*, these CpG islands are unmethylated. Our DNA methylation results (Fig. 8) correspond to these observations as we found that there is no methylation of Regions 1 to 6 in normal osteoblast cells.

WNT signaling is important in colorectal cancer (Ying *et al.* 2008, Hibi *et al.* 2009, Rawson *et al.* 2011), so we also looked at DNA methylation data available for colorectal cancer and the corresponding normal tissue. The findings showed that in most cases Regions 3, 4 and 5 was methylated in normal colon and colorectal cancer. However, the degree of methylation in colorectal cancer was higher than in corresponding normal colon sample. Also, extensive methylation in Regions 1-6 was present in the tumor tissue sample from patient 81 when compared to the normal (Fig. 6). Results suggest that there is variation in DNA methylation in Regions 1 to 6 in colorectal tumors but not normal cells that express *WNT5A*, but it appears that CpG islands associated with Regions 3 and 4 are often methylated. These results are consistent with

our findings in that we found Regions 3, 4 and 5 to be completely methylated in SaOS-2 cells.

#### *WNT5A Intron 1 is Aberrantly Methylated in Osteosarcoma SaOS-2 Cells*

We performed bisulfite sequencing of the intron 1 CpG Regions 1 to 6 of DNA from normal human osteoblast cells and osteosarcoma SaOS-2 cells. There are no other published reports of similar analyses. The results obtained for osteoblasts show that all CpG Regions 1-6 are unmethylated. In osteosarcoma, Regions 1 and 2 are unmethylated, Regions 3, 4 and 5 are methylated and Region 6 is partly methylated. 1-3 clones were analyzed for each region. However, for this study only one clone for osteosarcoma CpG Region 6 was analyzed. More clones are currently being prepared for sequencing. The methylated part of Region 6 contains the Exon 1B start of transcription and this methylation could be the reason for the repression of promoter B transcription. Regions 1 and 2 are unmethylated and near promoter A. Possibly the high transcriptional activity of promoter A is somehow responsible for the lack of Region 1 and 2 methylation. The methylation of Regions 3, 4 and 5 could be due to other mechanisms not directly related to promoters A and B. These possibilities are discussed below.

Cancer cells tend to show general global hypermethylation and gene specific hypomethylation (Shen *et al.* 2013). *WNT5A* is one gene that is subject to epigenetic modification by DNA methylation (see Introduction). Our data confirm this general conclusion as we found that *WNT5A* is methylated at specific CpG islands in

osteosarcoma SaOS-2. We do not know if this pattern of methylation is unique to SaOS-2 cells or more common to all osteosarcoma tumor cells. This is currently being explored in our lab. From the bioinformatics data it appears that certain CpG islands in *WNT5A* are prone to methylation, particularly Regions 3 and 4. It has been suggested that aberrant methylation in cancer could be a result of stochastic effects, where DNA methylation takes place randomly in retrotransposon sequences such as LINE (Long Interspersed Elements) and SINE (Short Interspersed Elements), which are present throughout the human genome (Estécio et al. 2012 and 2010 ). Insulator proteins such as the CTCF and USF 1/2 also influence patterns of DNA methylation (Van Roon *et al.* 2011). Also DNA methylation could be affected by other sequences such as the proximity of active promoters. CpG Regions 1 and 2 are located near promoter A, which is highly active in osteosarcoma SaOS-2 cell line. The active promoter A could have an effect on Regions 1 and 2, keeping them free of methylation. This would include particular histone modifications associated with active transcription including H3K4me3 and H3K9ac and the RNA polymerase transcription itself.

CpG Regions 3, 4 and 5 are likely not involved in promoter A or B regulation/expression due to their locations within intron 1. The aberrant methylation of these regions could be due to the presence of LINE and SINE sequences in the regions. However, examination of the data available in the UCSC Genome Browser for LINE and SINE repeats shows that there are no such repeats in the regions. Another reason for the aberrant methylation of these regions could be due to insulator proteins, e.g. CTCF and

USF 1/2 . An ENCODE search for insulator proteins CTCF and USF 1/2 in osteoblasts showed that intron 1 includes binding sites for the insulator protein CTCF (Fig. 11). Possibly the type of sequence of Regions 3 and 4, plus the presence of insulators lead to aberrant methylation during transformation.

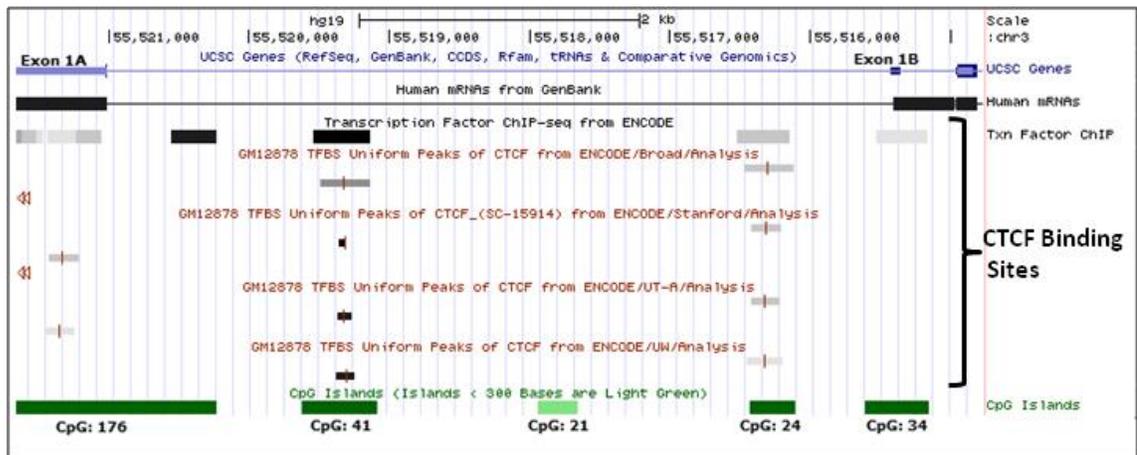


Figure 11. Insulator protein CTCFs are present in the *WNT5A* intron 1 region. Screen shot from *WNT5A* genomic region of ENCODE search for CTCF.

CpG region 6 is associated directly with promoter B and it is only partially methylated. The reason for this partial methylation could be due to histone modification associated with active transcription e.g. H3K4me3 and H3K9ac. A NCBI epigenomics search for histone modifications in osteoblast samples showed that there are histone modifications H3K4me1 and H3K36me3, which are related to transcriptional activation near Region 6. Also, CTCF insulator protein binding site is present near region 6 (Fig. 12). The histone modifications, insulator proteins combined with distinct sequences of

the region may contribute to a distinct pattern of DNA methylation during transformation.

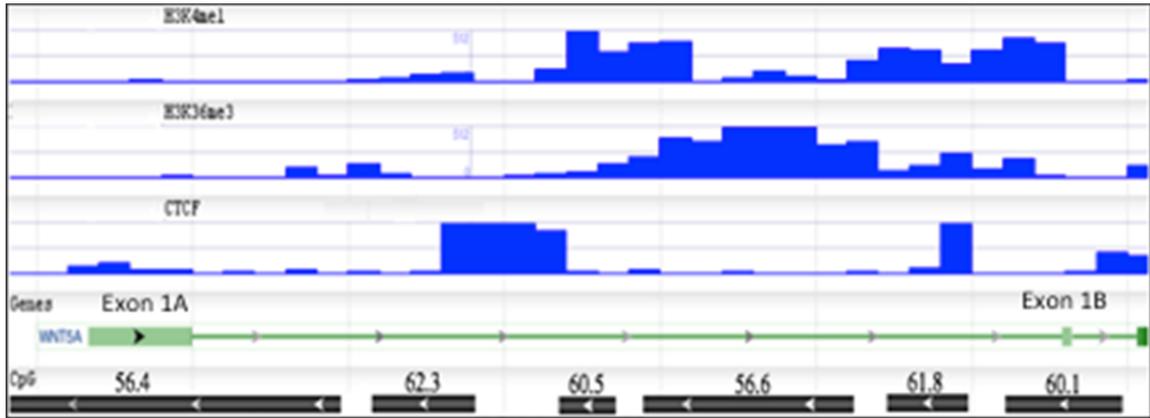


Figure 12. H3K4me1 and H3K36me3 and CTCF insulator protein binding sites in WNT5A Intron 1 region.

*Promoter B is Activated by 5-Aza-cytidine and Region 6 is Prone to Demethylation*

Promoter B is inactive or active at a low level in SaOS-2 cells. In comparison, transcripts from promoter B are highly expressed in normal osteoblast cells. My results indicate that promoter B inactivity in SaOS-2 cells is associated with DNA methylation. To test for causality, we treated the SaOS-2 cells with 5- Aza-cytidine that inhibits DNA methyl transferases (DNMT's), enzymes responsible for the addition of the methyl groups and causes hypomethylation.

My hypothesis is that 5-Aza-cytidine should increase promoter B transcript levels and decrease methylation of the promoter B associated CpG islands. Indeed, we found that SaOS-2 cells that had been treated with different concentrations of 5-Aza-cytidine

increased promoter B transcript levels, there was a slight decrease of promoter A transcript levels.

When a concentration of 1  $\mu\text{M}$  5-Aza-cytidine was used promoter B transcripts increased 120 fold. Treatment with 5  $\mu\text{M}$  and 10  $\mu\text{M}$  resulted in lower amounts of promoter B transcripts when compared to 1  $\mu\text{M}$ . This might be because 5-Aza-cytidine causes global DNA methylation, other genes may be activated that are affecting promoter B transcription. Also, 5-Aza-cytidine increases cytotoxicity and can cause cell death.

Bisulfite sequencing analysis of the CpG islands indicated that Regions 3, 4 and 5 were resistant to demethylation. Only a few demethylation changes were detected. However, of the clones that have been analyzed for Region 6, two showed more extensive demethylation. Of the 5 CpG's, 4 were demethylated in the two clones. This result indicates that Region 6 is more prone to de methylation. Currently more Region 6 clones are being analyzed.

Region 6 most certainly associated with promoter B expression. As previously discussed, Region 6 is likely to have a distinct chromatin state in comparison to Regions 3, 4 and 5, due to its inclusion of promoter B transcription start site and Exon 1 B sequences. This may also explain why this region is more prone to demethylation. The finding that Region 6 is only partially methylated suggest that it is also protected from methylation. It will be interesting to determine the types of histone modifications associated with the Region 6 in comparison to Regions 3, 4 and 5.

### *Future Plans*

To further study the role of DNA methylation in *WNT5A* Promoter B expression in osteosarcoma, DNA methylation profiles in the CpG islands of other osteosarcoma cell lines such as the U2OS cell line should be determined. We should also quantify the levels of promoter A and promoter B transcripts in these cell lines. Another experiment would be to obtain patient samples of osteosarcoma and adjacent normal tissues and measure the promoter A and promoter B transcript levels and analyze the amount of DNA methylation present in the CpG islands. Together these studies will answer the questions: Is there a specific, defined pattern of DNA methylation associated with osteosarcoma tumors or is DNA methylation variable across the *WNT5A* intron 1 region? And, is promoter B transcription always reduced in osteosarcoma tumors? Additional studies will need to focus on the functional distinction between *WNT5A* protein isoforms A and B, derived from the unique transcripts of promoter A and

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