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Regulation Of Survivin Gene Expression In The Human Endometrium And Endometrial Cancer

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CHAPTER 1: INTRODUCTION

Endometrial Cancer

In the United States, endometrial cancer is the leading cancer of the female reproductive tract. There are 40,100 new cases and 7,470 deaths from endometrial cancer estimated for 2008 (47). The average five year survival rate for endometrial cancer is 84% however, this figure is substantially lower in patients diagnosed with late stage, advanced disease and much higher for patients diagnosed in early stage disease (47). Endometrial cancer (EC) has been associated with several risk factors including obesity, diabetes, hypertension, previously documented occurrence of hereditary non-polyposis colorectal cancer (HNPCC), and heightened exposure to estrogen (25). As of yet, there has not been a dependable molecular predictor of endometrial cancer occurrence in women with these predisposing factors. The goal of our lab is to identify genes that are aberrantly expressed in EC and may serve as molecular biomarkers of EC progression. One candidate protein that we are exploring as a biomarker of EC progression is the cell survival protein survivin.

Survivin

Discovery

Survivin (*BIRC5*) was first cloned and characterized in Diego Altieri's laboratory at Yale University School of Medicine in 1997. At the time, the Altieri laboratory was studying components of the coagulation cascade and their contribution to vascular injury. Specifically, they were interested in the mechanism by which activation of the coagulation cascade through protease factor Xa binding to its receptor effector cell protease-1 (EPR-1) not only promoted thrombin formation but also promoted cellular growth (2, 5, 87). To identify other genes homologous to EPR-1, they conducted hybridization screening of the

human P1 genomic library with the cDNA of EPR-1 and found a new gene in the same locus but on the DNA strand opposite of EPR-1 (Figure 1).

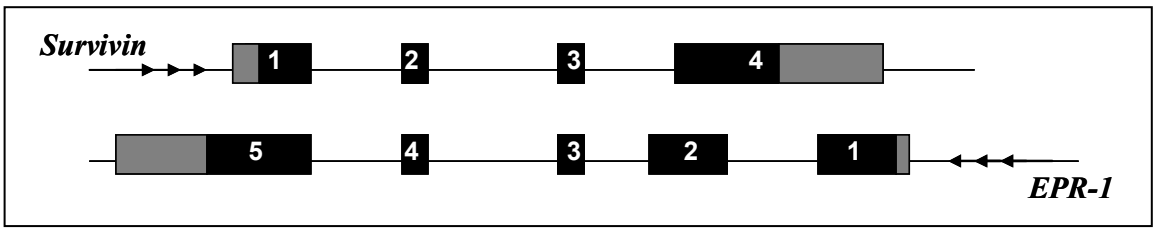

Figure 1: Map of the *survivin* gene

The *survivin* and *EPR-1* genes are located on opposite strands at the chromosome 17q25 locus. Arrows indicate the direction of transcription, numbered boxes represent exons, gray shading indicates the UTR.

Sequence analysis of this new gene predicted the formation of a unique 1.9kb transcript that would form a 142 amino acid protein, unrelated to EPR-1, with a molecular weight of 16,389kD that were later identified in transformed lymphoid cell lines by Northern blotting and immunoblotting. Blast analysis of the protein sequence indicated the presence of a baculovirus IAP repeat (BIR) domain that is the distinguishing feature of the IAP inhibitor of apoptosis family, thus the new protein was presumed to function as a survival protein and was named survivin (6).

Structure

The 14,796 nucleotide *survivin* gene is located on chromosome 17q25, contains a TATA-less proximal promoter, 4 dominant exons (1,2,3,4), 2 cryptic exons

(2B,3B) that are introduced by alternative splicing events and 3 introns. Five survivin transcripts have been identified which code for functional proteins (Figure 2). The predominant survivin transcript is made up of all four exons and encodes a 142 amino acid (aa) protein. It contains the BIR domain, and a carboxy-terminal coiled-coil (microtubule interacting) domain that directs survivin interaction with tubulin (6, 9).

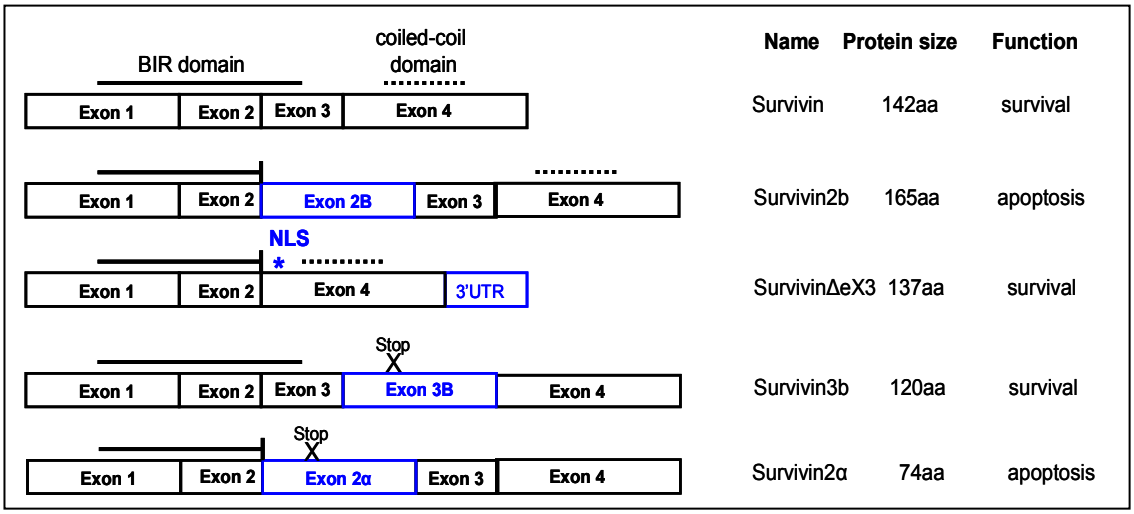

Figure 2: Schematic of Survivin Splice Variants

The *survivin* gene encodes for the primary survivin transcript as well as four splice variants. Survivin2B and survivin2α retain portions of intron2 while survivin3B retains part of intron3 and survivinΔeX3 is missing all of exon3. Horizontal bars indicate the BIR domain, vertical lines mark a truncated BIR domain, dashed lines indicate the coiled coil domain, an X indicates an in-frame stop signal and the blue asterisk indicates a unique nuclear localization signal.

The BIR and coiled-coil domains in the survivin protein are separated by the dimer interface. The survivin2B transcript includes a 69-bp cryptic exon 2B from intron 2 and

encodes a 165aa protein with a truncated BIR domain and an intact coiled-coil domain. The survivinΔeX3 transcript is missing all of exon3 resulting in a frame shift which incorporates part of the downstream 3' un-translated region (UTR) into the transcript. This variant encodes for a 137aa protein with a truncated BIR domain, a coiled-coil domain and a unique nuclear localization signal (NLS) which is homologous to the NLS found in steroid hormone receptors (66). Survivin2α includes a 197-bp insertion from intron 2 which creates a premature stop codon within the insertion. The resultant 74aa protein has a truncated BIR domain, and lacks the coiled-coil domain (12). Survivin3B includes a 165-bp cryptic exon 3B from intron 3 and encodes a 120aa protein that has been associated with increased cytoprotection (9, 53). The survivinΔeX3 protein retains the pro-survival function of the primary survivin protein however, the survivin2B and survivin2α proteins have a dominant negative effect and actually promote cell death (13, 78). Most studies are focused on the dominant survivin isoform as its expression is much higher than the splice variant isoforms and we will focus our discussion on just the dominant survivin protein isoform.

Function

Studies of survivin function over the last 12 years have yielded varied and controversial results. The presence of the BIR domain makes survivin the smallest member of the IAP gene family and suggests that this protein functions to inhibit apoptosis. This is supported by evidence that survivin overexpression in various mammalian cells leads to enhanced cell survival by inhibition of apoptosis whereas survivin depletion leads to vast and spontaneous apoptosis (4). This function of survivin was initially debated because the survivin protein lacks the CARD (caspase activation and recruitment domain) domain

present in other IAPs and there was no evidence that survivin could directly bind and inhibit caspase activation. This was resolved as later studies indicated that the only IAP family member that is capable of direct caspase inactivation is the largest family member, XIAP, thus the inability of survivin to directly inhibit caspases did not negate its potential role as an IAP (23, 24). However, survivin knockdown in worms did not indicate any deficiencies in apoptosis, instead the phenotype indicated defective mitosis. Similarly, survivin knockout mice are embryonic lethal due to improper microtubule formation and polyploidy (104). Subsequent studies show that survivin functions both in apoptosis and cell cycle regulation according to its subcellular localization (3).

Subcellular localization

Recent studies indicate that there are distinct "pools" of survivin. These pools of survivin represent the dominant survivin protein and are distinct from the splice variants. One pool of survivin functions in normal cells during cell division and localizes to several parts of the mitotic machinery as part of the chromosomal passenger complex made up of Aurora B kinase, Inner centromere protein (*INCENP*) and Borealin. Aurora B kinase phosphorylation of survivin at Thr117 alters survivin localization at the centromere to maintain proper chromosome alignment and segregation (31, 62), bipolar spindle formation (88), and completion of cytokinesis (104). While these survivin functions promote the fidelity of cell division, during chromosome alignment, the occurrence of a disattached kinetochore activates checkpoint proteins to arrest division and the cell is subsequently marked for apoptosis. Here survivin, when phosphorylated by CDC2/cyclin B1 at Thr34,

exhibits increased protein stability, associates with the kinetochore microtubule, stabilizes it and indirectly inhibits caspase activation thereby evading apoptosis (31, 71, 77).

A second pool of survivin localizes to the mitochondrial intermembrane space and mitochondrial matrix (21) and is associated with inhibition of apoptosis. Inside the mitochondria survivin is able to bind and sequester Smac (94), an XIAP inhibitor, preventing its release into the cytosol thus relieving XIAP from inhibition (15). Certain cell death stimuli can, through a mechanism that has not been elucidated, stimulate the release of survivin into the cytosol where it forms a complex with XIAP, stabilizes it against proteasomal degradation, and further enhances cell survival through caspase inhibition (20).

A third less understood pool of survivin exists in the nuclei of interphase cells especially in cancer cells. The function of survivin in the nuclei of interphase cells has not been elucidated but this population of survivin protein does not exhibit anti-apoptotic function (18) and is exported from the nucleus through an interaction with the Ran-GTP effector molecule Crm-1(54). X-ray crystallography indicates that the region of survivin that binds Crm-1overlaps the binding region of Borealin in the chromosomal passenger complex (49). This suggests that Crm-1 binding may prevent survivin from joining this complex.

Expression

Survivin's pattern of expression is also distinct from the other IAPs. Survivin is highly expressed during embryonic and fetal development and is overexpressed in virtually all tumor types (6, 60, 78). In contrast, survivin is transcriptionally silent in most highly

differentiated adult tissues. It is however, expressed in the normal brain, ovary and in the proliferative phase of the cycling human endometrium (33, 55) suggesting that it may play a role in normal endometrial physiology. Many groups have implicated a physiological role for survivin in the murine endometrium but the precise function of survivin in this tissue is unclear. High expression of survivin has been found in the endometrium of mice exhibiting defective implantation and subsequent pregnancy loss (35, 61). Loss of survivin expression has also been described in the decidua of interleukin 11 receptor alpha null mice which are infertile due to aberrations in decidualization and trophoblast invasion (35).

Transcriptional Regulation

Survivin contains a TATA-less promoter such that transcription is driven through two critical Sp1 sites at the -151 and -171 positions (Figure 3) (6, 7, 60). Survivin expression is tightly cell-cycle regulated through the several cell cycle dependent elements (CDE) and cycle homology regions (CHR) present in the survivin promoter that rapidly increase survivin expression during mitosis. Non-cell cycle mediated regulation of survivin has been observed in response to growth factor signaling (106), stimulation with hormones (30, 32, 72, 89) and cytokines (8). The survivin promoter also contains a validated p53 binding site that overlaps an E2F binding site that binds p53 and several E2F family members (50, 82).

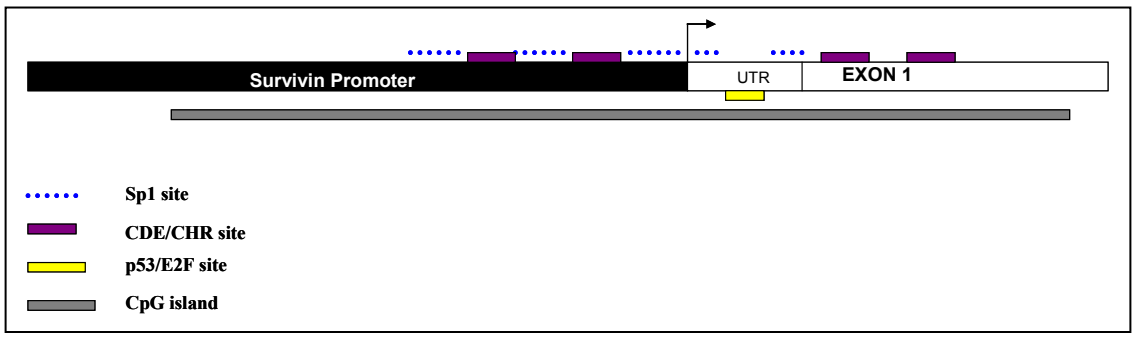

Figure 3: Map of the *survivin* promoter

Representation of the proximal survivin promoter with blue circles indicating the Sp1 sites, purple rectangles indicating the cell cycle regulation sites, yellow rectangle indicates the p53/E2F binding site and a gray rectangle indicates the CpG island.

The survivin promoter is GC rich with a characteristic CpG island. The observed frequency of the dinucleotide CG throughout the human genome is much lower than the mathematically expected frequency except in regions called CpG islands where there tends to be CG enrichment. The "p" in CpG refers to the phosphodiester bond linking the cytosine and guanine. The definition of a CpG island is a genomic region of greater than 200bp with a GC content greater than 50% and an observed/expected CpG ratio of 0.6 (36). The CpG island in the survivin promoter is 498bp in length with 51 CpG dinucleotides, 71% GC content and an observed/expected CpG ratio of 0.81(UCSC Genome Browser). This characteristic CpG island extends into exon 1 (Figure 3) and has been shown to be subject to epigenetic regulation during development and in response to DNA damage (6, 7, 28, 40, 60).

Epigenetics

Epigenetics is the study of heritable changes which affect gene expression but do not cause genotypic changes. This form of gene regulation involves reversibly changing the structure of chromatin surrounding coding regions of DNA such that gene promoters become physically permissive or inhibitory to transcriptional activation. Two well described types of epigenetic regulation are DNA methylation and histone tail modification.

The most common histone modifications are acetylation and de-acetylation of their amino terminal tails. Acetylation of the histone tails "opens up" the DNA allowing for recruitment of transcriptional machinery. Conversely, de-acetylation promotes tighter chromatin packaging and leaves the region repressive to transcriptional activation (64).

DNA methylation is the transfer of a methyl group from a methyl donor (S-adenosyl methionine) to the cytosine in a CG pair by a DNA methyl transferase enzyme which results in gene silencing. In normal cells, most non-promoter CG sites are methylated and promoter CG sites are un-methylated. A hallmark of cancer progression is an initial wave of global ***hypo***methylation whereby previously methylated CG sites in intergenic regions become unmethylated. This is followed by local ***hyper***methylation of CG sites in gene promoter regions. Hypermethylation, and therefore silencing, of tumor suppressors is a well documented phenomenon in the etiology of various tumor types (64).

The presence of a CpG island in the survivin promoter suggests that survivin gene expression may be regulated by DNA methylation. Since survivin is transcriptionally silent in most normal adult tissue, many groups speculated that the survivin promoter would normally be methylated. However, the methylation status of the survivin promoter has been analyzed in several tissue types an in most cases it has been reported that the survivin promoter is unmethylated in normal tissues (29, 44, 60, 74, 82, 111, 118, 119). One report showed that the survivin promoter was methylated in normal ovarian tissue but hypomethylated in ovarian tumors. Since the expression status of survivin is similar in the

endometrium as it is in the ovary, we speculated that DNA hypomethylation could explain survivin overexpression in endometrial cancer.

Purpose of project and rationale:

We observed that survivin was overexpressed in high grade endometrial tumors and hypothesized that DNA hypomethylation could be responsible for this overexpression. We conducted methylation analysis and determined that the survivin promoter was hypermethylated not hypomethylated in EC progression. To explain this seemingly paradoxical result, we speculated that DNA methylation could activate survivin expression in tumors by inhibiting the binding of a transcriptional repressor. In the subsequent work, we will present data supporting our hypothesis that DNA methylation of the survivin promoter functions to de-repress survivin expression in cancer cells by inhibiting the binding and repressive function of the tumor suppressor protein p53.

CHAPTER 2: SURVIVIN IS OVEREXPRESSED AND HYPERMETHYLATED IN ENDOMETRIAL CANCER.

Introduction

Molecular progression of endometrial cancer

Endometrial tumors are classified into two categories, Type I or Type II. Type I tumors are the most common, accounting for 80% of endometrial cancer cases. Histologically, these tumors are well-differentiated and endometrioid (resembling endometrial glands). These tumors are graded from 1-4 based on the level of cellular differentiation such that the most well-differentiated tumors are low grade (EC1) and have the best prognosis whereas high grade (EC3-4) tumors are poorly differentiated and have the worst prognosis (27, 90). Type I tumors commonly occur in pre- or peri-menopausal women who have a heightened exposure to estrogen and are generally preceded by endometrial hyperplasia (51). Common genetic mutations is the Type I tumors include the DNA mismatch repair enzymes (*MLH1, MSH2, MSH6*) (11), *KRAS* (56) , *PTEN* (73), and *CTNNB1* (β-Catenin) (65) whereas *TP53* mutations are extremely rare (<1%) (81). By contrast, Type II tumors are less understood, they are poorly differentiated and tend to be of the papillary serous, or clear cell types. These tumors are highly invasive and carry a poor prognosis (10). They tend to occur in older post-menopausal women and are not linked to hyper-estrogenic signaling or endometrial hyperplasia (75, 105). Genetically, these tumors commonly have alterations in *TP53* (86). We will focus our studies on the Type I, more common tumor type.

Epigenetic control in Endometrial Cancer

In Type I tumors, epigenetic studies have linked hypermethylation to the loss of expression of DNA repair enzymes MLH1 and O6-methylaguanine DNA methyltransferase (MGMT), the tumor suppressors PTEN, p53 and TIG1, the progesterone receptor, the β-catenin/Wnt signaling regulator APC, the transcription factor C/EBPα, and the differentiation gene HoxA10 (34, 90, 117, 120). Alternatively, hypomethylation has been described as a contributing factor for oncogene over-expression in EC. The oncogene PAX2 (90, 113) and the metastasis promoting gene S100A4 (114) were shown to be overexpressed and hypomethylated in aggressive endometrial cancer. Based on these observations, we hypothesized that survivin would be similarly hypomethylated in endometrial cancer. In this chapter we will explore 2 questions: 1.) Is survivin overexpressed in Type I endometrial tumors? 2.) What is the methylation status of the survivin promoter in normal endometria and endometrial tumors?

Materials and Methods

Human Endometrial Tissue

All human tissue samples were obtained from patients under protocols approved by Institutional Review Boards at the University of Texas Health Science Center and MD Anderson Cancer Center.

Gene expression was analyzed in fresh frozen tissues from 71 endometrioid adenocarcinoma samples obtained at the time of hysterectomy and submitted to the Department of Pathology, MD Anderson Cancer Center. Diagnoses were confirmed following light microscopic examination of H&E-stained slides by a gynecologic pathologist

at MD Anderson Cancer Center (Houston, TX). Tissues were frozen in liquid nitrogen and stored at -80 C.

Gene expression was analyzed in postmenopausal endometrial tissues obtained from a selected subset (n = 10) of a large group of healthy postmenopausal women (n = 210) participating in a clinical trial of estrogen replacement therapy (ERT). These 210 women were randomly divided into three groups receiving one of the following three treatments: 1) placebo; 2) conjugated estrogens (2:1, wt/wt) of estrone sulfate and α-equilin (EES; 0.625 mg/d, Wyeth Research, Philadelphia, PA); or 3) Premarin (0.625 mg/d; Wyeth Research) for 6 months under conditions approved by the Human Ethics Committee of Escola Paulista de Medicina Universidade Federal de São Paulo, Brazil. Tissues were frozen in liquid nitrogen and stored at -80 C.

DNA methylation analysis was conducted on normal tissues derived from baseline endometrial biopsies from women with HNPCC mutations who were enrolled in a chemoprevention trial of depot medroxyprogesterone acetate (DEPO, Pfizer, New York, NY) versus combination oral contraceptives (0.03mg ethinyl estradiol/ 0.3mg norgestrel, Wyeth-Ayerst) for prevention of endometrial cancer. These women have a 71% lifetime risk of developing endometrial cancer, compared to 3% for the general population and tend to develop cancer 15 years earlier than patients from the general population(1, 11, 22, 39, 48, 63, 112). The women enrolled in the study (n=68) either have known HNPCC mutations or fulfill Amsterdam criteria for HNPCC but have a histologically confirmed “normal” endometrium such that there is no evidence of endometrial cancer or its precursor, endometrial hyperplasia. Endometrial pipelle biopsies (n=10) were obtained under

conditions approved by the institutional review board at the University of Texas M.D. Anderson Cancer Center. Tissues were frozen in liquid nitrogen and stored at -80 C. DNA methylation status was also analyzed in human endometrial cancer cells which were manually dissected from adjacent stroma in paraffin-embedded tumor sections using a 2.0 mm biopsy punch (Fray Products Corp.).

RNA extraction

Frozen tissues were homogenized in TriReagent® (Molecular Research Center) and RNA was precipitated with isopropanol, applied to RNeasy spin columns (Qiagen), eluted, and treated with RNase-free DNase for 30 min at 37 C, followed by heat inactivation at 75 C and storage at -80ºC.

Quantitative Real Time-RT PCR (QPCR)

Taq-Man assays utilize the 5' nuclease activity of Taq DNA polymerase and an assay specific fluorogenic probe as a means to quantify the abundance of a transcript. The probe is designed with a fluorescent reporter dye (FAM) on the 5' end and a quencher dye (BHQ1) on the 3' end. During the extension cycle, the DNA polymerase cleaves the reporter dye thereby releasing it from the quencher and subsequently a fluorescent signal is emitted and detected. The fluorescent signal from the unknown samples is compared to the signal measured from a standard curve of synthetic DNA (sDNA) oligos designed to represent the amplicon of each assay. The sDNA is serially diluted in 10-fold decrements to represent a 5-log scale of fluorescent signal plotted against a known sDNA quantity from which fluorescent signal from unknown samples (C_T) is interpolated into transcript quantity.

Taqman real-time quantitative assays for *survivin* and *18S rRNA* were developed using Primer Express software (Applied Biosystems) based on sequences from GenBank. The assays were developed and all QPCR reactions were completed at the Quantitative Genomics Core Laboratory (UT-Houston Medical School, Houston, TX, USA). The primer and probe sequences and accession numbers for all assays are listed in **Table1**.

Forty nanograms of RNA (10ng/μL) from each sample were reverse transcribed in triplicate on a 7700 format 96-well plate (ISC Bioexpress, Kaysville, UT) in 6 μL of reaction master mix containing 400nM assay-specific reverse primer, 500 μM deoxynucleotides, Stratascript buffer, and 10U Stratascript reverse transcriptase (Stratagene, Cedar Creek, TX). The assay of each sample also included a nonamplification control well which contained all reagents and RNA but was lacking the reverse transcriptase enzyme. The plate was incubated for 30 minutes at 50º followed by 20 minutes at 72º in a thermocycler. Afterwards, 40μL of PCR master mix containing 400nM assay specific primers, 100nM assay specific probe with a 5' 6-FAM (5-carboxyfluorescein) and a 3' Black Hole Quencher 1 (BHQ1), 5mM $MgCl_2$, 200μM deoxynucleotides, PCR buffer and 1.25U Taq polymerase was added to each well and amplified in an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) under the following cycling conditions: 95°C for 1 min, 40 cycles of 95°C for 12sec, and 60°C for 30 sec. The results were analyzed using SDS 1.9.1 software (Applied Biosystems) with SuperROX (BioSearch, Novato, CA) as a reference dye. The mean transcript levels for all assays were normalized to the housekeeping gene 18s ribosomal RNA transcript levels. Data are presented as a median ratio of (transcript/18s).

Assay (Type)	Primer & Probe Sequences	Accession#
Survivin (Taqman)	181+CCACTGCCCCACTGAGAAC 255-GGCTCCCAGCCTTCCAG 204+FAM-CAGACTTGGCCCAGTGTTTCTTCTGCT-BHQ1	NM_001168
18s rRNA (Taqman)	1335+CGGCTTAATTTGACTCAACAC 1401-ATCAATCTGTCAATCCTGTCC 1359+FAM-AAACCTCACCCGGCCCG-BHQ1	M10098
ApoB100 (Taqman)	13503+CCTTTGAGGTCTTATTCACGAAT 13579-AATGCAAGAAGAAAACCTAGGG 13554-FAM-ACTTCTCTGGACATTGGCCTAGACA-BHQ1	NT_022184
Survivin (MSP)	(M)FWD+GGCGGGAGGATTATAATTTTCG, (M)REV-CCGCCACCTCTACCAACG (U)FWD+GGTGGGAGGATTATAATTTTTG, (U)REV- CCACCACCACCACCTCTAC	U75285
Survivin (pyrosequencing)	FWD+GGYGGGAGGATTATAATTTT REV-biotin-AAAAAAAACTACCAAACAAAAAAC SEQ+GTTTTTATTTTTAGAAGGT	U75285
Ki67 (Taqman)	3323+AAGTTCACACGGACGTCAG 3391-GATGCTCTTGCCATCTCC 3347+FAM-ACCACGCACACGCACAGAGAG-BHQ1	NM_002417
cMYC (Taqman)	1479+ACACATCAGCACAACTACGC 1540-CTCTTGGCAGCAGGATAGTC 1501+FAM-CGCCTCCCTCCACTCGGAA-BHQ1	NM_002467
Survivin (gel-shift)	Sense-GCCTAAGAGGGCGTGCGCTCCCGACATGCCCCGCGGCGCG Anti-sense-TGGCGCGCCGCGGGGCATGTC GGGAGCGCACGCCCTCTTA	U75285
Survivin (ChIP)	Fwd-ACTACAACTCCCGGCACA Rev-AGAGATGCGGTGGTCCTTGAGAAA	U75285
Nek2 (UPL)	1372+AGTGCAAGGACCTGAAGAAAAG 1417-TCAATATCTGACAGGGCTTGAG UPL#44 (universal probe library, Roche applied sciences)	NM_002497.2
HMGB1 (UPL)	5+GAGTAATGTTACAGAGCGGAGAGA 56-AATGTACTGCAATGGCTGTGAG UPL#75	NM_002128.4
CDC25C (Taqman)	39+ CCGTAACTTTGGCCTTCTGC 111- CAGCTCTGCCTTCCGACTG 86-FAM-CCAACGTCGGACTCAGAGTCTTCCCT-BHQ1	NM_001790

Table 1: QPCR Primer and Probe Sequences

DNA isolation

DNA was isolated according to TriReagent® protocol by phenol and chloroform separation, ethanol precipitation, and solubilization in 8mM NaOH. HEPES was used to adjust the pH to 8.4 prior to bisulfite treatment.

Copy Number Variation (CNV) Assay

Forty nanograms of genomic DNA from normal endometrial samples and from endometrial tumors isolated as described above were amplified by QPCR in a 25µL reaction volume consisting of: 1X PCR buffer, 5mM $MgCl_2$, 200µM dNTPs, 400nM assay specific primers, 100nM assay specific probe and 1.25U Taq polymerase, and detected in an ABI Prism 7700 as described above. Survivin levels were normalized to the levels of the housekeeping gene ApoB that is located on chromosome 1 and does not exhibit CNV in tumors. The ApoB assay was designed within intron 10. Relative gene copy number was determined using the using the comparative delta Ct method by the formula $2^{-(\Delta\Delta Ct+/-SD)}$ where one normal sample is designated as the calibrator to which all other samples are compared. $\Delta\Delta Ct = (Ct\ ApoB_{calibrator} - Ct\ Survivin_{calibrator}) - (Ct\ ApoB_{sample} - Ct\ Survivin_{sample})$. The Ct (cycles to threshold) is defined as the point (cycle) at which the fluorescence level rises above baseline (threshold). The mean relative copy number for all the normal samples was then compared to the mean relative copy number for all the tumor samples.

Bisulfite Treatment of DNA

Sodium bisulfite has been used extensively in studies of DNA methylation because of its ability to preferentially deaminate unmethylated cytosines and convert them to uracils. The uracil is then read as an adenine during polymerase based amplification methods and the final result is a sequence with a C to T mutation. Methylated cytosines are protected from the deamination reaction and therefore no conversion occurs. This allows for discrimination between methylated and unmethylated cytosines. Bisulfite modification of 2μg of DNA was performed by using the EZ DNA Methylation-Gold Kit™ (Zymo Research Corp., Orange, CA) according to manufacturer protocol. DNA was heat denatured for 10 minutes at 98ºC and treated with a sodium bisulfite conversion reagent for 2.5 hours at 64ºC in a thermocycler. This step serves to hydrolytically deaminate all unmethylated cytosines into uracils whereas methylated cytosines remain as cytosines. The resultant uracil from the deamination reaction is concomitantly sulphonated by the sodium bisulfite and must therefore be desulphonated. This is achieved by addition of the sample to a provided spin column and treatment with an alkali desulphonation buffer. The DNA is then washed and eluted through a series of spins and stored at -20ºC until subsequent analysis.

To determine if bisulfite conversion was successful, two sets of primer pairs were designed downstream of the Survivin promoter in Exon 4 where none of the cytosines are subject to methylation and should therefore all convert to thymidine. One primer pair was designed to reflect the bisulfite converted condition where all cytosines are converted to thymidines and the second primer pair was designed to reflect the untreated condition for which all cytosines maintain identity. Each bisulfite treated DNA sample was PCR amplified in parallel with each set of primers and the PCR product was subject to gel

electrophoresis and visualized by ethidium bromide staining. Only, samples for which there was a bright "converted" band were included for further analysis. Primer sequences are:

Converted	**Unconverted**
F: 5'-GTGTTGTTGGTAATAGTGGTT	F: 5'-GTGCTGCTGGTAACAGTGG
R: 5'-CATAAAATCCAAACACATTCA	R: 5'-CATGAGGTCCAGACACATTCA

Metylation specific PCR (MSP)

Bisulfite treated DNA from 5 normal endometrial tissue and 15 endometrioid adenocarcinomas was PCR amplified in parallel in a 10µL reaction volume under the following conditions: 1X PCR buffer (16.6 mM ammonium sulfate, 67 mM Tris-HCL pH 8.8, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol), 0.5mM dNTPs, 200nM specific methylated or unmethylated PCR primer pairs and 0.5U HotStart Taq DNA polymerase (Qiagen). Thermocycler condition were: 15min. at 95ºC, 35 cycles of 30sec. at 95ºC, 30sec. at 55ºC and 30sec. at 72ºC followed by a final extension step of 10min. at 72ºC. PCR product was subject to gel electrophoresis on a 6% 0.5X TAE polyacrylamide gel and visualized by ethidium bromide staining.

Bisulfite Pyrosequencing™ Analysis

PCR reactions were carried in 50 µl reaction volume including 2 µl bisulfite treated DNA, 16 mM (NH4)2SO4,67 mM Tris-HCl (pH 8.8), 1 mM 2-mercaptoethanol 2 mM MgCl2, 0.125 mM dNTP, 1 unit Taq polymerase, and 200 nM primers. Results were quantitated using the PSQ HS 96Pyrosequencing System (Pyrosequencing Inc) at the UCLA Sequencing Core in the Department of Human Genetics. Three of the samples were spiked

with 100%, 25% and 10% in vitro methylated DNA to monitor the efficiency of the pyrosequencing reaction. The assay is biased against methylation such that the measured percentages for the 100%, 25% and 10% methylated samples were 32%, 6% and 3% respectively. We conducted linear regression analysis and generated a standard curve with a 99% correlation coefficient to correct for this assay bias.

Sss1 in vitro Methylation

To validate the ability of the MSP and Pyrosequencing assay to detect methylation, DNA samples were methylated in vitro using the CpG Methylase M.SssI (New England Biolabs, Ipswich, MA) which is isolated from a strain of *E. coli.* containing the methylase gene from *Spiroplasma* sp. strain MQ1. DNA was methylated according to manufacturer protocol. The DNA sample is incubated for 2 hours at 37ºC with Sss1 methylase, the supplied buffer, and the methyl donor S-adenosyl-methionine (SAM). Sss1 non-specifically transfers a methyl group from SAM to any CpG quartet. The reaction is stopped by heating at 65ºC for 20 minutes and the DNA is purified by phenol extraction followed by ethanol precipitation and then subject to bisulfite treatment and PCR amplification.

Results

Survivin is overexpressed in endometrial cancer

Using quantitative RT-PCR (QPCR) we quantified survivin transcript levels from 10 normal post menopausal endometrial samples and 71 endometrioid adenocarcinoma samples. Survivin mRNA levels progressively increased from normal to grade 3 tumors (median: normal= 0.06, EC1= 0.42, EC2= 0.89, EC3= 1.32) and were significantly increased 14.9 fold in grade 2 and 22.7 fold in grade 3 endometrial tumors compared to normal samples (**Figure 4**). This suggests that survivin overexpression is selective towards high grade, poorly differentiated tumors. This finding is consistent with RT-PCR and immunohistochemistry data from other laboratories (26, 27, 57, 99).

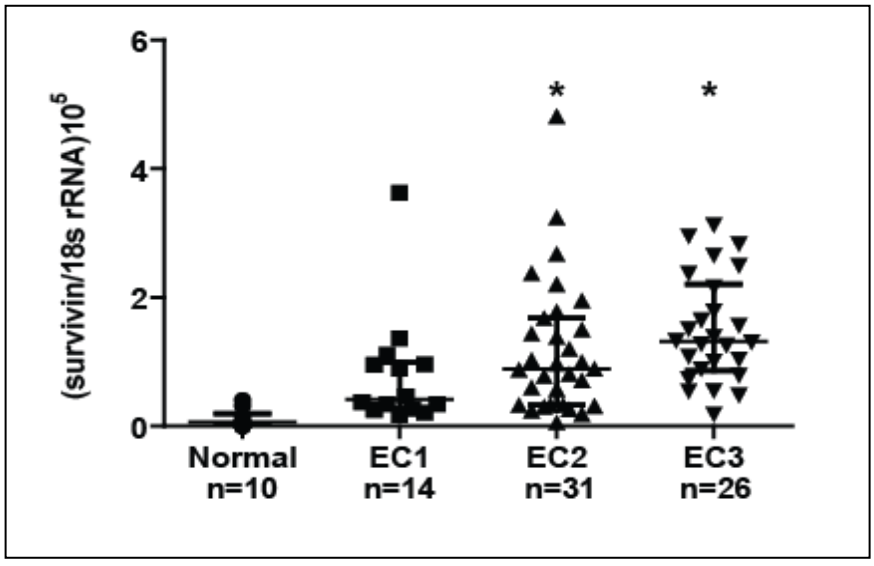

Figure 4: Survivin is overexpressed in endometrial cancer
QPCR analysis of survivin expression in normal endometria and grade1 to grade 3 endometrial tumors (EC1-EC3). Data is presented as a median ratio of survivin/18s rRNA*(10^5) ± interquartile range. *p<0.001 by Kruskal-Wallis non-parametric two-way ANOVA and Dunn's post test

Nabilsi et al, Oncogene 2009

Survivin overexpression in endometrial cancer is not due to gene amplification

Two common causes of oncogene overexpression in cancer are activating SNPs (single nucleotide polymorphisms) and gene amplification. To date there are no known activating SNPs reported for survivin in any pathology (3), however survivin gene amplification has been reported in neuroblastoma (98). To determine if survivin overexpression in endometrial cancer is due to amplification of the *survivin* gene, we conducted a copy number variation (CNV) assay. Survivin gene copy number was quantified by QPCR in 5 normal and 14 tumor samples and normalized to the copy number of the gene apolipoprotein B100 (ApoB). ApoB is a known single copy gene (per haploid genome) located on chromosome 2 (42). A requisite for normalizing survivin levels to ApoB levels is that the efficiency of the two assays must be similar. We generated a standard curve for each assay by quantifying gene copy levels in 5 serial half dilutions of DNA quantity (50ng, 25ng, 12.5ng, 6.25ng, and 3.125ng), plotting the detected C_T values as a function of the DNA quantity, and conducting linear regression analysis (**Figure 5a and 5b**). The efficiency of each assay is reflected by the slope of the line generated by the standard curve such that 100% efficiency= -3.33. The slopes of the survivin assay and ApoB assay were both -3.43 thus we were confident in the utility of ApoB to normalize for survivin. We observed no difference between the survivin copy number detected in normal tissue (mean= 1.32) compared to endometrial tumors (mean= 1.33); p=0.83 (**Figure 6**). We concluded that the overexpression of survivin observed in endometrial tumors is not due to gene amplification.

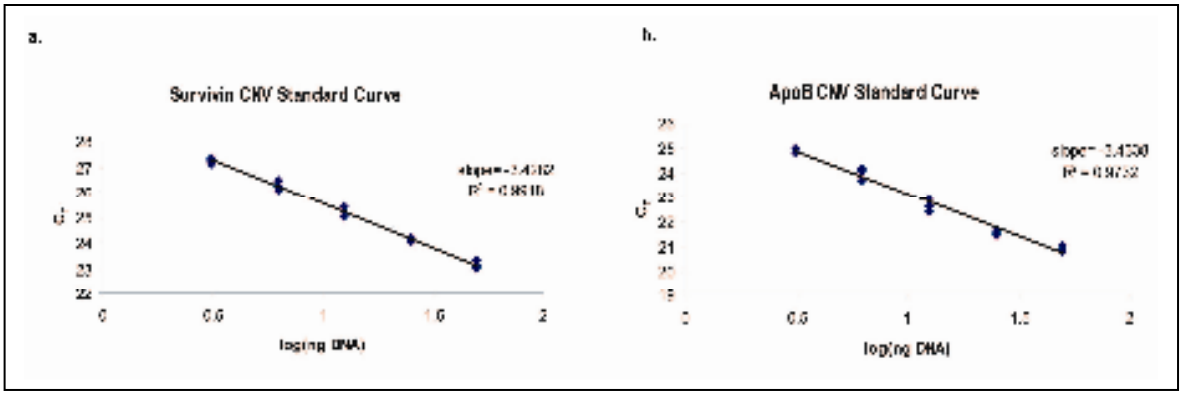

Figure 5: Comparison of efficiency for survivin and ApoB CNV QPCR assays

Regression analysis was used to determine the QPCR assay efficiency for (a.) survivin and (b.) ApoB to detect gene copy number as a function of a known DNA quantity. The efficiency is determined by the slope of the line such that 100%= -3.33. Both assays had a slope= -3.43.

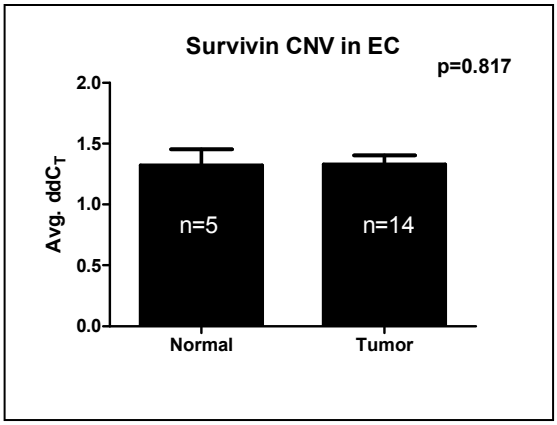

Figure 6: Survivin overexpression in endometrial cancer is not due to gene amplification

CNV analysis of 5 normal endometria and 14 high grade endometrial tumors. Data are presented as the relative ratio of survivin gene copy number normalized to ApoB gene copy number. Details for the relative quantification equation are given in the materials & methods. An unpaired Student's t test was used for statistical analysis.

Examination of the survivin promoter indicated the presence of a canonical CpG island which suggests that survivin may be regulated by DNA methylation. Given the expression status of survivin, we hypothesized that the survivin promoter was methylated in the normal endometrium then becomes hypomethylated throughout EC progression.

The *survivin* promoter is hypermethylated in endometrial cancer

Surprisingly, analysis of the survivin promoter with methylation specific PCR (MSP) showed that normal endometrial samples were completely unmethylated whereas methylation progressively increased from low grade to high grade endometrial tumors correlating with increased survivin expression (Figure 7a). In an independent set of normal and endometrial tumor samples, we used bisulfite pyrosequencing to analyze the methylation status of 12 CpG sites within the 5' untranslated region of Exon 1. We observed that only 11% of the CpGs have at least 10% methylation in normal samples compared to 33% of the CpGs being methylated in tumors, p=0.012 (Figure 7b). Our MSP and pyrosequencing data both showed increased methylation that correlated with survivin over-expression; this suggested that DNA methylation of the survivin promoter may inhibit the binding of a transcriptional repressor. The tumor suppressor protein p53 is a well documented repressor of survivin (28, 43, 69) and the p53 binding site in the survivin promoter contains three internal CpG sites. Pyrosequencing data showed that none of the normal samples are methylated at the p53 binding site whereas 64% of the tumors are methylated at this site (Figure 7c). Furthermore, expression analysis of these tumors indicated that survivin expression increased with increasing methylation at the p53 binding

site. We hypothesized that DNA methylation could inhibit p53 binding thereby relieving survivin of p53 mediated transcriptional repression.

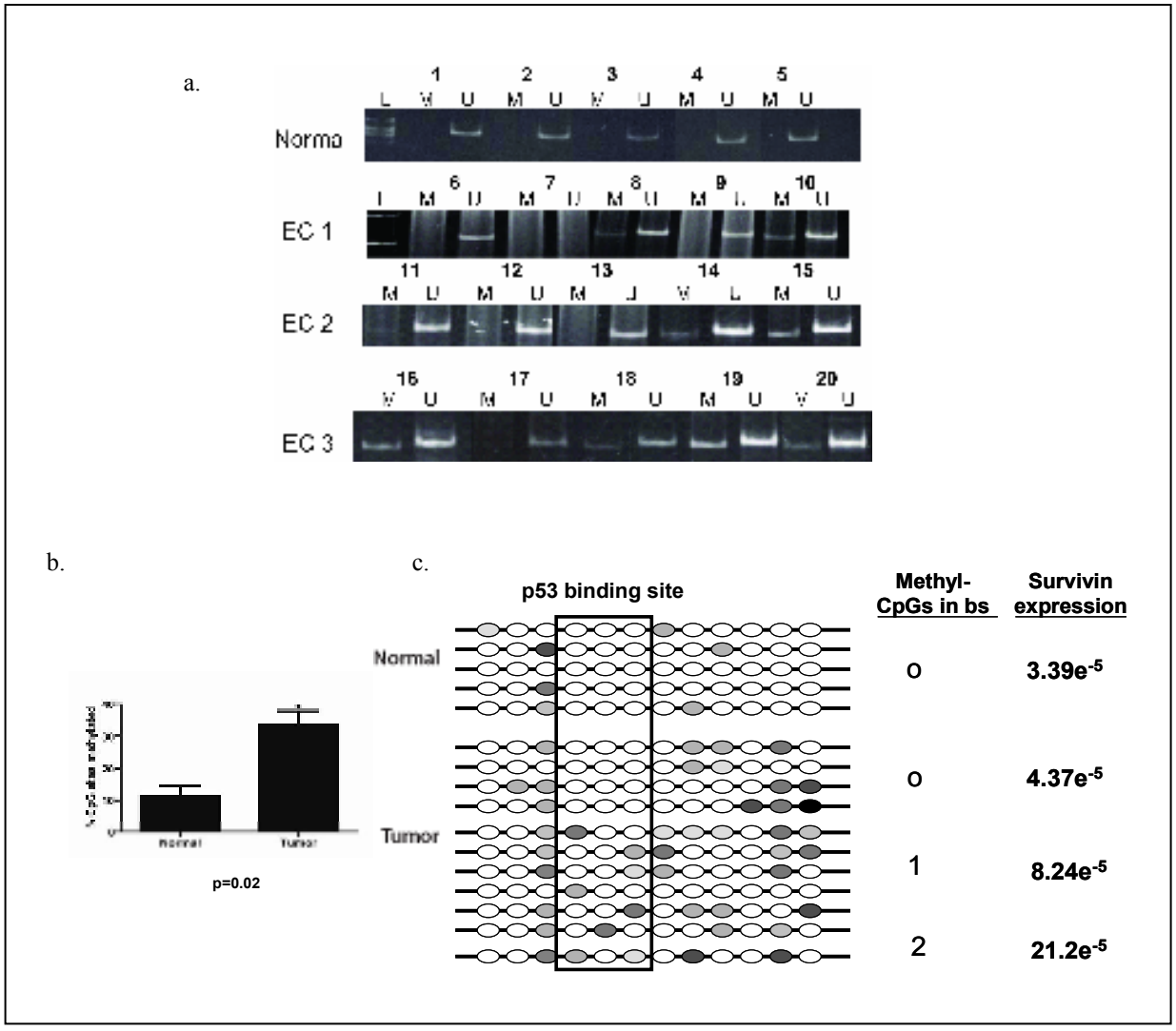

Figure 7: Methylation analysis of the survivin promoter

(a) MSP analysis of 5 normals and 15 EC tumors. Each number represents 1 DNA sample amplified with primers which detect methylated (M) or un-methylated (U) survivin sequences. (b.) Pyrosequencing analysis of DNA from normals (n=5) and EC tumors (n=11). Mean % of CpG sites methylated in normal vs. tumor samples depicted graphically; p=0.02 determined by Mann-Whitney unpaired t-test. (c.) Each circle represents a CpG site, unfilled circles represent unmethylated sites and filled circles indicate methylation. Increased darkness corresponds to increasing % methylation. Tumors are stratified according to the number of CpG sites methylated in the p53 binding site (bs) and the corresponding mean survivin levels are indicated. Data are presented as a mean ratio of survivin/18srRNA.

Modified from *Nabilsi et al Oncogene 2009*

Discussion

We have explored the expression and methylation status of survivin in the human endometrium. We observed by QPCR that survivin is overexpressed in endometrial tumors compared to normal endometria and that this overexpression is not caused by survivin gene amplification. Another potential cause of survivin overexpression in EC could be mediated by RNAi (RNA interference). RNAi occurs when complementary RNA strands bind and form a double stranded RNA which will results in either (1.) a secondary structure that inhibits the translational machinery from translating the transcript or (2.) activation of the endoribonuclease Dicer to bind and degrade the RNA duplex. Since the survivin transcript lies antisense to the EPR-1 transcript, there could be an RNAi mediated interaction between them which would alter gene expression. It was reported that both transcripts are expressed in various hematological cell types but EPR-1 expression was dominant in normal cells

while survivin expression was low and survivin expression was dominant in several cancer cell lines and leukemias while EPR-1 expression was low (79, 115). Furthermore, transfection of EPR-1 into colon cancer cells drastically reduced survivin expression through an RNAi mediated mechanism (115). To determine if a similar mechanism exists in endometrial cells, we designed a Taqman QPCR assay with an EPR-1 strand specific probe and measured EPR-1 transcript levels in normal endometria and in endometrial tumors. If survivin expression is modulated by EPR-1 levels then we expected to find high levels of EPR-1 in normal tissue and progressive loss of EPR-1 throughout EC progression. We found that EPR-1 transcript was not detectable in any normal tissue or tumor samples, nor in any endometrial cancer cell lines (data not shown). We concluded that aberrant EPR-1 expression does not contribute to survivin overexpression in EC.

Epigenetic studies utilizing two different bisulfite methodologies, MSP and pyrosequencing in two independent sets of normal endometria and endometrial tumors indicated that the survivin promoter is unmethylated in normal endometrial tissue and is hypermethylated in EC. While this pattern follows the hallmark pattern of DNA methylation whereby most gene promoters are unmethylated in normal tissue then become methylated in tumors, this was an incredibly surprising result since hypermethylation is generally associated with gene silencing, not gene activation. Mechanistically, one potential explanation for increased methylation correlating to increased expression is that the presence of methylation at this promoter could inhibit the binding of a transcriptional repressor. The most well-documented repressor of survivin expression is the tumor suppressor protein p53

and the p53 binding site in the survivin promoter lies within the hypermethylated CpG island.

Our working model is that in the normal endometrium, where the p53 binding site is unmethylated, p53 is able to bind and repress survivin expression (Figure 8a.). In contrast, in endometrial tumors, where the p53 binding site is methylated, p53 binding is inhibited thus relieving survivin of p53 mediated repression (Figure 8b). The inhibition of p53 binding can be achieved either directly by the presence of the methyl groups themselves or through the recruitment of methyl binding proteins to the methylated DNA. Furthermore, the p53 binding site in the survivin promoter overlaps an E2F binding site which has been shown to bind the transcription factor E2F1 and activate survivin expression. The E2F binding site has only 1 CpG site and is therefore less likely to be inhibited by DNA methylation as p53 which contains 3 CpG sites. Thus, a third scenario is that DNA methylation at the p53/E2F binding site results in inhibition of p53 binding and preferentiates E2F1 binding which further increases survivin expression (Figure 8c). In the next chapter, we will validate that p53 represses survivin in our model system.

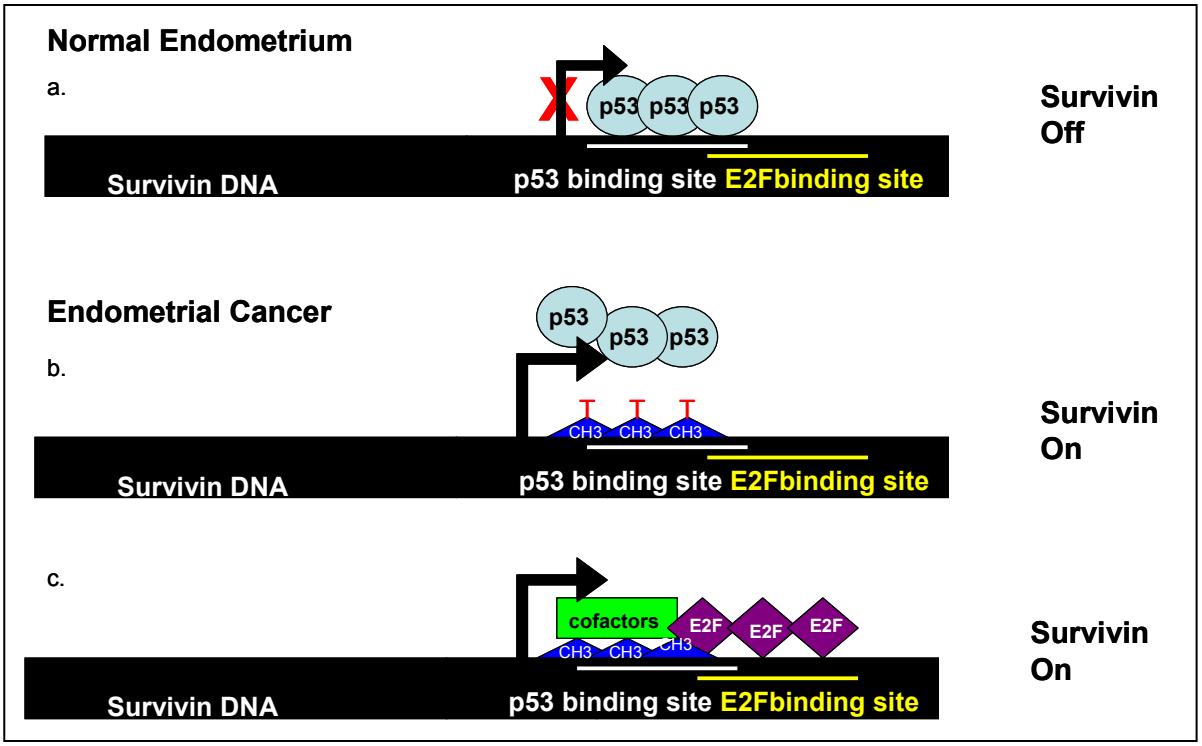

Figure 8: Model for activation of survivin by DNA methylation

CHAPTER 3: P53 REPRESSES SURVIVIN EXPRESSION

Introduction

"If genius is the ability to reduce the complicated to the simple, then the study of p53 makes fools of us all" (109)

The tumor suppressor protein p53 is a transcription factor that is activated following a variety of cellular stress signals. This signal can include several types of DNA damage, telomere shortening, hypoxia, mitotic spindle dysfunction, and temperature shock (108). These stress signals are detected by various proteins that modify the p53 protein or its negative regulator, MDM2. MDM2 is a ubiquitin ligase that blocks p53 transcriptional activity and mediates its degradation. After a stress signal, MDM2 polyubiquitylates itself thus targeting its own destruction, and subsequently increases the half-life of p53 from minutes to hours allowing for its accumulation, modification and action (110). The stabilized p53 protein (phosphorylated, methylated and/or acetylated at specific serine or lysine residues) binds as a tetramer to its targets' p53 binding sites and activates or represses transcription in order to arrest a cell for repair of DNA damage or, if the damage or insult is beyond repair, signal for apoptosis (58, 59). The result of p53 signaling is to maintain the genomic integrity of the cell and thereby prevents cancer. Perturbations of proper p53 function increases a cell's tumorigenic potential. This is evident by the fact that nearly 50% of all tumors contain p53 mutations (85).

p53 is a well documented survivin repressor and examination of the survivin promoter indicates that the p53 binding site contains 3 internal CpG sites which gain

methylation in endometrial tumors (Figure 4c). We suspect that methylation at the p53 binding site will inhibit p53 from binding to and repressing survivin. Several approaches have been used to demonstrate survivin repression by p53 including UV irradiation (43), doxorubicin treatment (28, 43) and p53 over-expression (69) in a variety of cell types but this regulation has not been examined in endometrial cells. In this chapter we will validate that p53 represses survivin in endometrial cancer cells.

Materials and Methods

Cell culture

Ishikawa endometrial cancer cells (ATCC) were maintained in RPMI1640 medium (Gibco) supplemented with 10% FBS, 1U/mL penicillin/ 1ug/mL streptomycin, and 10mM Hepes. HCT116*wt* and HCT116 *p53*$^{-/-}$ colon cancer cells (a generous gift from Dr. Bert Vogelstein) were maintained in McCoy's 5a growth medium (Gibco) supplemented with 10% FBS and 1U/mL penicillin/ 1μG/mL streptomycin. All cells were maintained in a humidified 37ºC incubator with 5% CO_2.

Drug treatment

Cells were grown to 60% confluency then treated with 0.5μM, 1μM or 2μM doxorubicin (Sigma) or vehicle (sterile water) as indicated for 48hrs.

Western Blot

Whole cell lysate

Cells were lysed by incubation for 1 hour at 4°C in 100 µL Triton lysis buffer [1% Triton X-100, 150 mmol/L NaCl, 25 mmol/L Tris (pH 7.5), 1 mmol/L glycerol phosphate, 1 mmol/L sodium fluoride, and 1X Complete Mini Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN)]. Lysates were centrifuged for 10 minutes at 14,000 x *g* at 4°C. Supernatants were saved and boiled for 5 minutes with SDS-PAGE sample buffer (50 mmol/L Tris-HCL, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 5% ß-mercaptoethanol). Samples then resolved by SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes. Membranes were incubated overnight at 4°C with primary antibodies specific for survivin (Cell Signaling Technology), p53 (Calbiochem) and β-actin (Millipore). Membranes were incubated with species-specific secondary antibodies then visualized by chemiluminescence.

Nuclear protein isolation

Cells were scraped into 1X PBS on ice then resuspended in a 0.5% Triton lysis buffer (10mM HEPES-KOH, 1.5mM MgCl2, 10mM KCl, 0.5mM DTT, and 1X Complete Mini Protease inhibitor cocktail (PIC)). Following centrifugation the supernatant was removed and the remaining nuclear pellet was resuspended in a 25% glycerol buffer (20mM HEPES-KOH, 420mM NaCl, 1.5mM MgCl2, 0.2mM EDTA, 0.5mM DTT and 1X PIC) and kept on ice for 30 minutes with agitation. Following centrifugation the nuclear protein was collected and the western blot conducted as described above.

Plasmids

We obtained a GFP-tagged p53 overexpression construct (Clontech, pp53-EGFP) from Dr. Donehower's laboratory at Baylor University. We mutagenized nucleotide 580 in the p53 coding sequence from C to T so that the mutant protein will have a phenylalanine instead of leucine at amino acid 194. This residue is in the DNA binding domain of p53 so the L194Fmutant is incapable of binding to DNA (69). Mutagenesis was conducted according to the Statagene quick-change mutagenesis kit with the primers:

L194F+: CCCCTCCTCAGCATTTTATCCGAGTGGAAG

L194F-: CTTCCACTCGGATAAAATGCTGAGGAGGGG

Successful mutagenesis was confirmed by DNA sequencing.

We obtained a survivin-luciferase construct from Dr. Mien Chi Hung at M.D. Anderson Cancer Center and subcloned 980bp of the survivin promoter into a CpG-free-luciferase vector (a kind gift from Dr. Michael Rehli at the University of Regensburg in Germany). This vector has been modified such that all of the CG dinucleotides in the vector backbone have been removed (52). We also obtained a p53-luciferase reporter (luciferase driven by several repeats of p53 binding sites) from Dr. Russell Broaddus at M.D. Anderson Cancer Center to confirm that our GFP-tagged p53 protein but not our L194F mutant can sufficiently activate transcription of p53 target genes. We co-transfected each reporter with an internal control vector of Renilla luciferase driven by the thymidine kinase promoter (TK-Renilla).

Luciferase Assay

Cells were trypsinized and seeded to 60% confluency in a 60mm plate then co-transfected with either the p53-luciferase or the survivin-luciferase constructs and TK-Renilla as an internal control for transfection efficiency. The next day, transfected cells were trypsinized and re-seeded into 24-well plates, allowed to recover for four hours then either treated with drug or transfected with the wt-p53-GFP or L194F-GFP vectors. Forty-eight hours after treatment, cells media was replaced with 1X PBS and cells were harvested in Promega passive lysis buffer. Lysates were then incubated with Promega Dual-Glo luciferase substrate for 10 minutes according to manufacturer protocol and transferred to polystyrene tubes for photon emission measurement. Each sample is measured in duplicate for 10 seconds with a Monolight 2010 luminometer. Following firefly luciferase activity measurement, a second reagent was added to each sample to quench the firefly-luciferase signal and to allow for measurement of Renilla luciferase activity following the same detection parameters. Data are presented as a mean ratio of firefly/Renilla luciferase in relative units (RU).

Results

Survivin is repressed by endogenous p53

To validate that p53 represses survivin expression in endometrial cells, we treated Ishikawa endometrial cancer cells with the drug doxorubicin (adriamycin). Doxorubicin is a chemotherapeutic agent that acts as a topoisomerase II (topoII) poison. TopoII binds to and unwinds DNA during transcription. Doxorubicin intercalates into DNA and stalls TopoII thereby activating a DNA damage response which marks the cell for apoptosis. p53 is one of the proteins activated by doxorubicin and treatment of cells with doxorubicin to induce the nuclear accumulation of p53 is a common technique used to study endogenous p53 function and gene regulation (38, 76, 80, 93, 100). Ishikawa cells are a well-differentiated endometrioid adenocarcinoma cell line that harbor a silent mutation in p53 that function as wild-type (103).

Immunoblot analysis showed a dose dependent increase in the nuclear accumulation of p53 following 1uM and 2uM doxorubicin (doxo) treatment that correlated with decreased survivin levels in Ishikawa cells (Figure 9a). Similarly, Ishikawa cells transfected with a survivin-luciferase reporter showed a significant dose dependent decrease in survivin promoter activation following doxorubicin treatment (0.5uM doxo= 37% repression, 1uM doxo= 69% repression, $p<0.05$, and $p<0.005$ respectively) (Figure 9b).

To confirm that the observed repression of survivin following doxorubicin treatment is dependent on p53, we treated wild-type and p53-null HCT116 colon cancer cells with

doxorubicin and conducted immunoblot analysis. We observed that survivin was repressed in the wild-type cells, but not in the p53-null cells following doxorubicin treatment (Figure 10a).

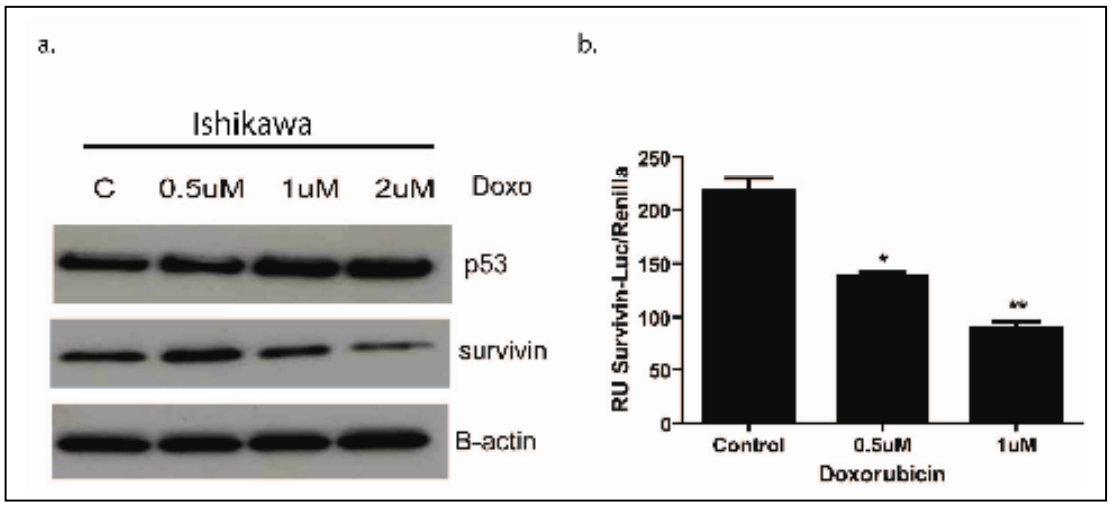

Figure 9: endogenous p53 represses survivin in endometrial cancer cells
Ishikawa cells were treated with doxorubicin (doxo) for 48 hrs. then harvested for (a.) immunoblot analysis or (b.) luminescence measurement. Control cells were treated with vehicle. B-actin serves as a loading control. *$p<0.05$, **$p<0.005$ by ANOVA followed by Bonferroni ad hoc post test.

Modified from *Nabilsi et al Oncogene 2009*

This finding is consistent with previous reports that repression of survivin by doxorubicin is mediated through p53 (28). E2F1 has been reported to be induced by doxorubicin (68) and serves as a control for drug induction in both wild-type and p53-null cells. Furthermore, transfection of the HCT116 cells with the survivin-luciferase reporter followed by doxorubicin treatment also showed decreased survivin promoter activation in response to

doxorubicin in the wild-type cells but not in the p53-null cells (Figure 10b) indicating that the repressive effect of doxorubicin on the survivin promoter is dependent on p53.

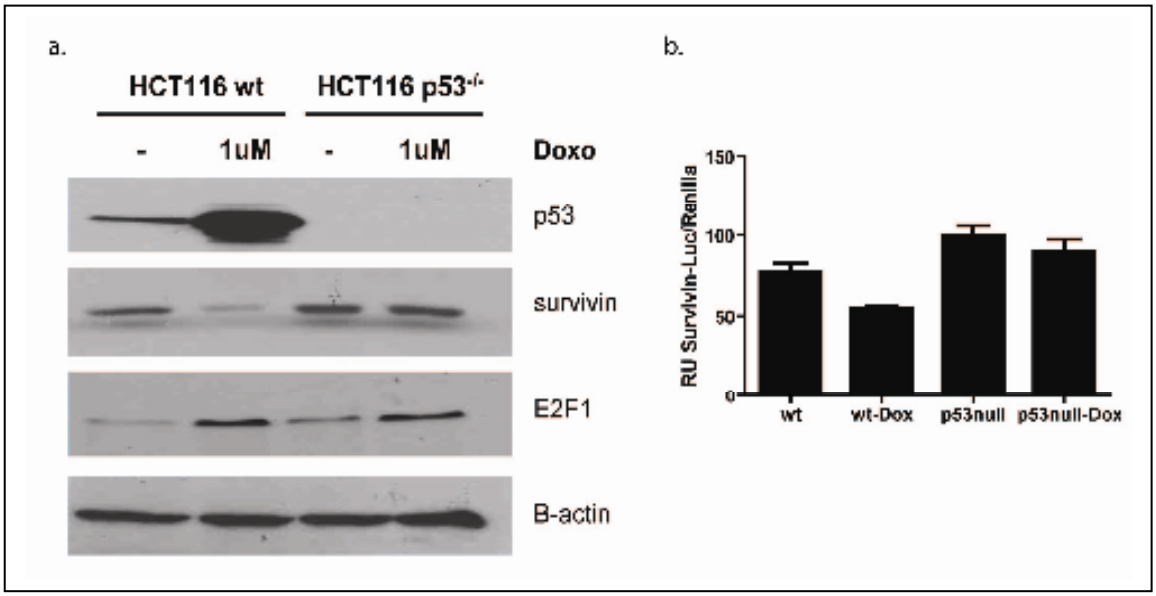

Figure 10: Doxorubicin mediated repression of survivin is dependent on p53
Wild-type and p53-null HCT116 colon cancer cells were treated with 1μM doxorubicin for 48 hrs. then harvested for (a.) immunoblot analysis or (b.) luminescence measurement. Control cells were treated with vehicle. B-actin serves as a loading control and E2F1 serves as a control for drug induction.

Modified from *Nabilsi et al Oncogene 2009*

Survivin repression by exogenously expressed p53 is dependent on DNA binding

We have shown that activation of endogenous p53 represses survivin protein expression and promoter activity. Next we wanted to determine if expressing exogenous p53 would repress survivin and if this repression was dependent on DNA binding. We obtained a

p53-GFP expression construct (a kind gift from Dr. Donehower) and mutagenized it to generate an L194F mutant that does not bind to DNA (69). This is one of the common "hot spot" mutations of p53 in human tumors and is the endogenous p53 mutation in T47D breast cancer cells (103). To confirm that wt-p53-GFP but not L194F-GFP can bind to and activate p53 target genes, we transfected a p53-luciferase reporter (luciferase driven by several repeats of p53 binding sites) with either wt-p53-GFP or L194F-GFP into p53-null HCT116 cells. We observed a 16-fold increase in p53 activity in response to wt-p53 expression but no change in response to the L194F DNA binding mutant indicating that wt-p53-GFP but not L194F-GFP can activate p53 target genes (Figure 11a).

To determine the effect of exogenously expressed p53 on survivin protein, we transfected wt-p53-GFP and L194F-GFP into Ishikawa cells and conducted immunoblot analysis. We observed that transfection of 2ug of the wt and mutant vectors resulted in equivalent expression of p53 but only the wt-p53 protein caused repression of survivin, not the DNA binding mutant (Figure 11b). Taken together, these data confirm that p53 represses survivin in endometrial cancer cells and that this repression is dependent on DNA binding.

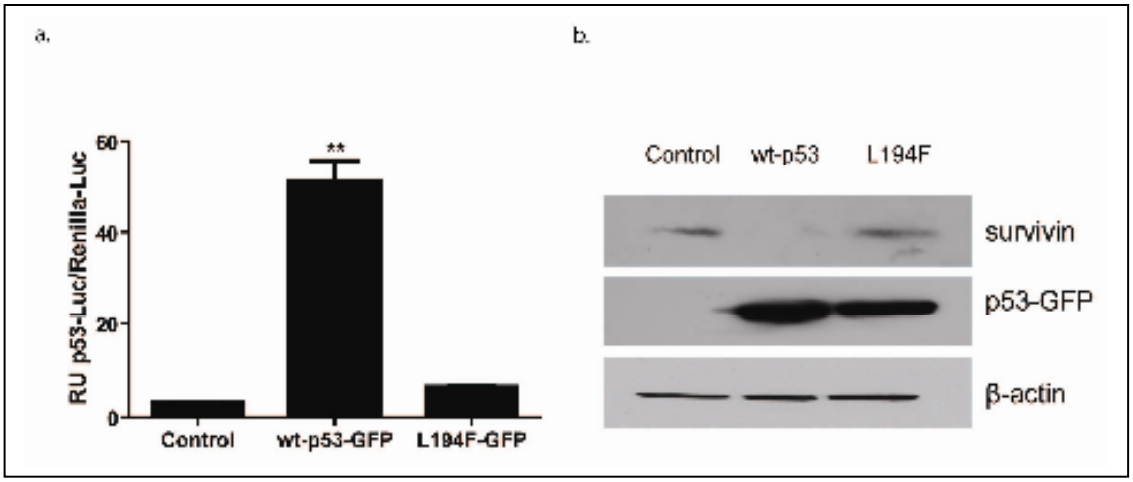

Figure 11: exogenous wt-p53 represses survivin in endometrial cancer cells
(a.) HCT116 p53-null cells were co-transfected with a p53-luciferase reporter and wt-p53-GFP or L194F-GFP then harvested for measurement of luminescence. n=3, **p<0.005 by ANOVA followed by Bonferroni ad hoc post test. (b.) Ishikawa cells were transfected with wt-p53-GFP or L194F-GFP and harvested for Immunoblot analysis. B-actin serves as a loading control.

Discussion

We have confirmed that survivin is repressed by p53 in endometrial cancer cells through induction of endogenous p53 protein by doxorubicin treatment and by expression of exogenous p53 protein. This finding is consistent with data reported by several groups in various model systems. It is interesting that p53 seems to exert a "basal" regulation of survivin expression. Most p53 target genes require not only the presence of p53 but also some form of activation of p53 to cause any changes in gene expression. For example, conditional "knock-in" of p53 into transgenic p53 knockout mice requires further activation of p53 by UV treatment in order to observe changes in c-Myc expression (67). However in Ishikawa cells, expression of wt-p53 through transient transfection was sufficient to cause survivin repression without any further p53 activation (Figure 11). This is also evident by the higher baseline survivin expression and promoter activity in the wild-type HCT116 cells compared to the p53-null cells (Figure 10). It is tempting to speculate that the increased presence of survivin in cells is so threatening to genomic stability that p53 is primed to repress this protein.

One of the oncogenic stimuli for p53 activation is c-Myc overexpression signaling through p19/ARF. Perhaps survivin is similarly not only regulated by p53 but is also capable of activating p53 in an auto-inhibitory fashion? I am not aware of any reports indicating that survivin can elicit a post-translational modification of p53 (i.e. phosphorylation, methylation, acetylation) but this may explain this unique activation-independent phenomenon. It would be interesting to transfect survivin cDNA into cells and to see if there is any effect on p53 stabilization.

Another interesting observation is that the basal activity of the survivin promoter in the luciferase experiments is quite high. Although this is consistent with previous reports, it is curious that survivin promoter activity is so robust in cancer cells without any external stimulus. It would be interesting to see if altering the amount of serum in the cell culture media has any effect on the baseline activity levels or if it is independent of growth stimuli. We have shown that treating Ishikawa cells with 1uM doxorubicin causes a 69% repression of survivin-luciferase activity however the equivalent treatment in HCT116 wild-type cells only caused a 30% repression. We believe this is because the baseline expression of survivin-luciferase in the HCT116 cells is much higher than in the Ishikawa cells and therefore dampened the effect. Without normalizing to TK-Renilla, the baseline activity of survivin-luciferase in Ishikawa cells is approximately 100,000 units whereas in HCT116 cells it is 600,000 units. Normalizing these data brings both figures down to the 100-200 R.U. range indicating that TK-Renilla is also highly expressed in the HCT116 cells compared to the Ishikawa cells. This suggests that the overall transfection efficiency is much

higher in the HCT116 cells than in the Ishikawa cells which may explain the differences in basal activity between the two cell types.

We have confirmed that survivin is repressed by p53 in our model system. In the next chapter we will explore the effect of DNA methylation on the ability of p53 to regulate survivin expression.

CHAPTER 4: DNA METHYLATION INHIBITS P53 MEDIATED SURVIVIN REPRESSION

Introduction

The current understanding of gene regulation by DNA methylation in cancer progression is that once a tumor suppressor gene's promoter gets methylated, DNA methyl binding proteins get recruited to the site of methylated DNA and inhibit the binding of the transcriptional machinery to that gene's promoter. In many cases, the methyl binding proteins recruit histone modifiers (ie histone deacetylases) which modify the chromatin to be repressive to transcription (64). Evidence for DNA methylation inhibiting the binding of specific transcription factors to DNA is scarce. This is surprising since methylation interference (and protection) assays have been widely used in the past to determine the DNA binding regions of various proteins.

The methylation interference assay was developed by the Walter Gilbert laboratory to study the specific interacting regions of *E. coli* RNA polymerase with DNA (92). In most cases this involved methylating guanine and adenine residues in the major groove of the DNA helix and looking for interference with DNA-protein binding however, the protocol was expanded to include cytosine methylation interference and many publications cite cytosine methylation interference of DNA-protein interactions (17, 45, 91). This cytosine methylation interference has been largely overlooked in the field of cancer epigenetics. More recently however, it has been published that DNA methylation of the *hTERT* (human telomerase) promoter activates its transcription by inhibiting the binding of CTCF (CCCTC binding factor), an hTert repressor (37, 83, 84). Similarly, gel shift studies indicate that DNA methylation can inhibit the binding of various E2F family members in a promoter specific context (14).

We have observed a correlation between increased survivin expression and increased survivin promoter methylation. We have also validated that p53 represses survivin expression in HCT116 colon cancer cells. In this chapter we will explore 3 questions: 1.) Does DNA methylation regulate survivin gene expression (or do they just correlate)? 2.) Is regulation of survivin by DNA methylation dependent on p53? 3.) Does DNA methylation of the survivin promoter inhibit p53 binding?

Materials and methods

Cell culture

HCT116*wt* and HCT116 *p53*$^{-/-}$ colon cancer cells (a generous gift from Dr. Bert Vogelstein) were maintained in McCoy's 5a growth medium (Gibco) supplemented with 10% FBS and 1U/mL penicillin/ 1μG/mL streptomycin. All cells were maintained in a humidified 37ºC incubator with 5% CO_2.

Drug treatment

HCT116 cells were seeded to 20% confluency overnight then maintained in 2μM or 200nM decitabine (5-Aza-2-deoxycytidine) (Sigma) as indicated or vehicle for 4 days. Due to the instability of the drug, media was changed daily with fresh drug application.

Methylation Specific PCR (MSP), QPCR and Western Blot

Following the drug treatment described above: genomic DNA, RNA and protein were isolated and analyzed by MSP, QPCR and western blot respectively, as described in Chapter 2.

Cell cycle assay

A commonly used dye for cell cycle analysis is propidium iodide. The dye intercalates into the major groove of double-stranded DNA and produces a fluorescent adduct that can be excited at 488 nm with an emission around 600 nm. The amount of signal reflects the DNA content of a cell and the stage of the cell cycle can then be inferred. Cells with 2N (diploid) DNA content are likely in G1, cells with 4N DNA content are in G2/M and cells with DNA content in between 2N and 4N are at some point of replication in S-phase. HCT116 cells were treated with decitabine or vehicle as indicated for 4 days then harvested for cell cycle analysis. Cells were trypsinized and washed in 1XPBS then resuspended in propidium iodide buffer containing 50 μg/mL propidium iodide, 0.1% Triton-X 100, and 0.1% sodium citrate in 1X PBS. Samples were stored at 4°C for 2hrs. then vortexed and analyzed for DNA content by flow cytometry in a Guava® Personal Cell Analysis (PCA)-96 Flow Cytometer. Results were exported into excel for data analysis.

Gel-shift assay

Single stranded oligonucleotide probes representing the combined p53/E2F binding site in the survivin promoter were duplexed by heating to 95ºC then slowly cooling to 4ºC over 2 hrs. The probe is designed such that duplex will contain 3nt overhangs on the 3' ends to aid in increased labeling efficiency. Duplexed probe was column purified then methylated

according to SssI protocol described in Chapter 1. Methylation efficiency was monitored by digestion with the CpG methylation sensitive enzyme HinPI and polyacrylamide gel electrophoresis followed by ethidium bromide staining and visualization. We observed that 1hr. of incubation with the SssI methylase and S-adenosyl methionine (methyl donor) was sufficient to protect the probe from HinPI digestion (Figure 12a).

Methylated and unmethylated probe were then labeled with the Biotin 3' End DNA Labeling kit (Pierce Biotechnology) according to manufacturer's protocol. Biotinylation efficiency was estimated by dot blot analyses against control oligonucleotides. We observed an equivalent 25-30% labeling efficiency for both probes (Figure 12b). Binding reactions (RT, 20 min) contained 20ng purified p53 protein (Active Motif), buffer (10 mM Tris pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% TX-100, 2.5% glycerol), 1 µg poly(dI-dC), and 2 nM of methylated or unmethylated biotinylated survivin probe. Protein-DNA complexes were resolved on 5% Tris borate-EDTA gels, transferred to nylon membranes, and visualized utilizing the Chemiluminescent Nucleic Acid Detection Module (Pierce Biotechnology) according to manufacturer's protocol. Some reactions were preincubated for 10 min with 200-fold excess of unlabeled probe and/or an anti-p53 antibody (Active Motif) before adding biotinylated probe.

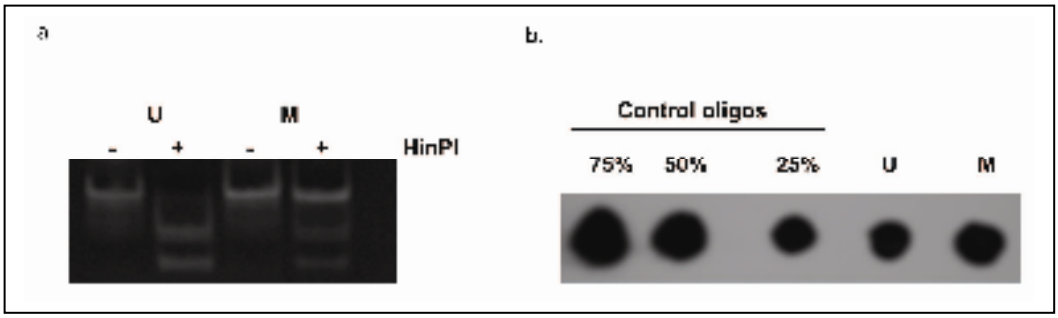

Figure 12: Gel-shift methylation and labeling controls

To confirm that the SssI reaction methylated the probe (a.) Unmethylated (U- lanes 1 and 2) and methylated (M- lanes 3 and 4) probes were incubated with or without the methylation sensitive enzyme HinPI. To confirm equivalent labeling of the probes (b.) a dot blot was conducted comparing U and M probes to control oligos of known biotin label percentages.

For the E2F1 gel shift, HCT116 p53-/- cells were transfected with a CMV-E2F1 overexpression vector (Addgene plasmid 10736- 408 pSG5L HA E2F1), and synchronized into S-phase by overnight serum withdrawal followed by 12hr. serum rescue to promote E2F1 accumulation in the nucleus. E2F1 enriched nuclear lysates were then prepared with NE-PER nuclear and cytoplasmic extraction kit (Pierce). Probes were incubated with 5µg nuclear lystae and analyzed as described above. Some reactions were preincubated for 10 min with 200-fold excess of unlabeled survivin probe or anti-E2F1 antibody (Santa Cruz) before adding biotinylated survivin.

Chromatin Immunoprecipitation (ChIP)

HCT116 cells were seeded to 20% confluency in two 100mM plates. Twelve hours after seeding, culture media was supplemented with 2uM decitabine or vehicle (1:10,000 diluted DMSO) and maintained for 4 days. Media was changed daily with fresh drug application. ChIP was carried out according to the IMPRINT ChIP kit protocol (Sigma). Briefly, cells were washed with 1XPBS, protein was crosslinked to DNA with formaldehyde

for 10 min., and the reaction was quenched with glycine for 5min. Cells were washed with ice-cold PBS, scraped and resuspended in nuclei releasing buffer followed by sonication buffer. Lysates were sonicated in a Sonics Vibra Cell Ultrasonic Processor at 40% output for 7 cycles of 30 second pulses (on ice) followed by 30 second rests on ice. An aliquot of sheared chromatin was reverse crosslinked, purified and fragments analyzed on a 1% agarose gel stained with ethidium bromide. The resulting DNA smear ranged from 1.5kB to 200bp with the average size around 600-700bp.

Lysates were incubated in stripwells coated with anti-p53 or anti-IgG antibodies, washed, reverse crosslinked and purified. The pulled-down DNA was then amplified with a WGA whole genome amplification kit (Sigma) and subject to 35 cycles of PCR amplification with survivin promoter specific PCR primers. Fifty ng of input DNA 5 ng (10%) input and 50ng of ChIP'ed DNA were incubated in a 25uL reaction with 1X JumpStart PCR buffer (Invitrogen), 5mM $MgCl_2$, 100nM primers, 200µM dNTPs and 0.25U JumpStart Taq (Invitrogen). Taq was activated for 5min. at 95º, reactions cycled at 95º for 30sec. 60º for 30sec., 72º for 30sec. followed by a 10min 72º extension then kept at -20º until loading in a 5% 1X TBE polyacrylamide gel. Electrophoresis was performed and products visualized by ethidium bromide staining. The image was captured with a digital camera and band densities quantified using AlphaEase Digidoc software. Band densities from the experimental samples were normalized to input band densities.

Statistical Analysis

Differences for QPCR decitabine studies were calculated by a two-way ANOVA followed by Tukey's ad hoc test.

Results

Demethylation of the survivin promoter results in p53-dendent survivin repression

To determine if survivin promoter methylation regulates gene expression, cells were treated with the demethylation agent 5-aza-2-deoxy-cytidine (decitabine) for four days to demethylate the survivin promoter. Decitabine is a nucleoside analogue. It resembles cytidine however, it is modified such that the carbon in the #5 position in the nucleoside which is the site of methylation is replaced with nitrogen (5-aza) which can not be methylated (Figure 13, downloaded from wikipedia) . The 5-aza-2-deoxy-cytidine gets incorporated into DNA in place of cytidine during replication therefore demethylating the genome. Also, the presence of the excess nucleoside attracts and binds to the DNA methyltransferase enzymes, thus acting as a DNA methyltransferase inhibitor, and further decreases the methylation of DNA.

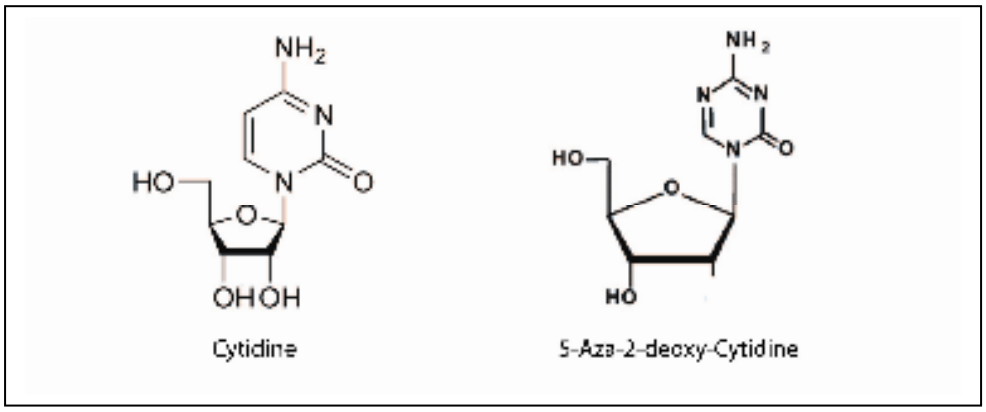

Figure 13: Structures of Cytidine and Decitabine

MSP analysis indicated that the survivin promoter in both the HCT116 wild type and p53 null cell lines was methylated but was unmethylated in Ishikawa cells (Figure 14). Four day decitabine treatment markedly (but not completely) reduced methylation of the survivin promoter in the HCT116 cells (Figure 15a). Immunoblot and/or QPCR analysis showed that survivin was significantly repressed following decitabine treatment in the wild-type cells (67%, p=0.05) but not in the p53 null cells (27%, non-significant) nor in the Ishikawa cells (10% at 1μM, no change at 2.5μM) (Figure 15b and 15c). The *c-Myc* gene is another oncogene which has been shown by others to be repressed by decitabine treatment in a p53 independent manner and was measured as a control for drug induction. We found that in both wild-type and p53-null HCT116 cells as well as in Ishikawa cells c-Myc was strongly repressed following decitabine treatment. These results show that survivin expression is regulated by DNA methylation because decitabine treatment repressed survivin in HCT116 cells that contain a methylated promoter but not in Ishikawa cells that contain an unmethylated promoter. Also, this repression is dependent on the presence of p53 because the p53-null HCT116 cells that also contain a methylated survivin promoter were not repressed by drug treatment.

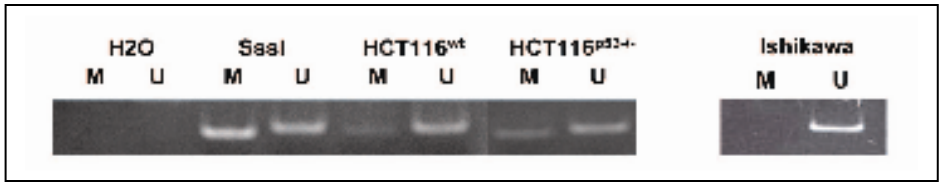

Figure 14: MSP analysis of the survivin promoter in cell lines
Bisulfite treated DNA from HCT116 cells and Ishikawa cells were incubated with MSP primers for methylated (M) or unmethylated (U) survivin DNA as described in Chapter 2. Water and SssI methylated DNA samples were included as negative and positive controls respectively.

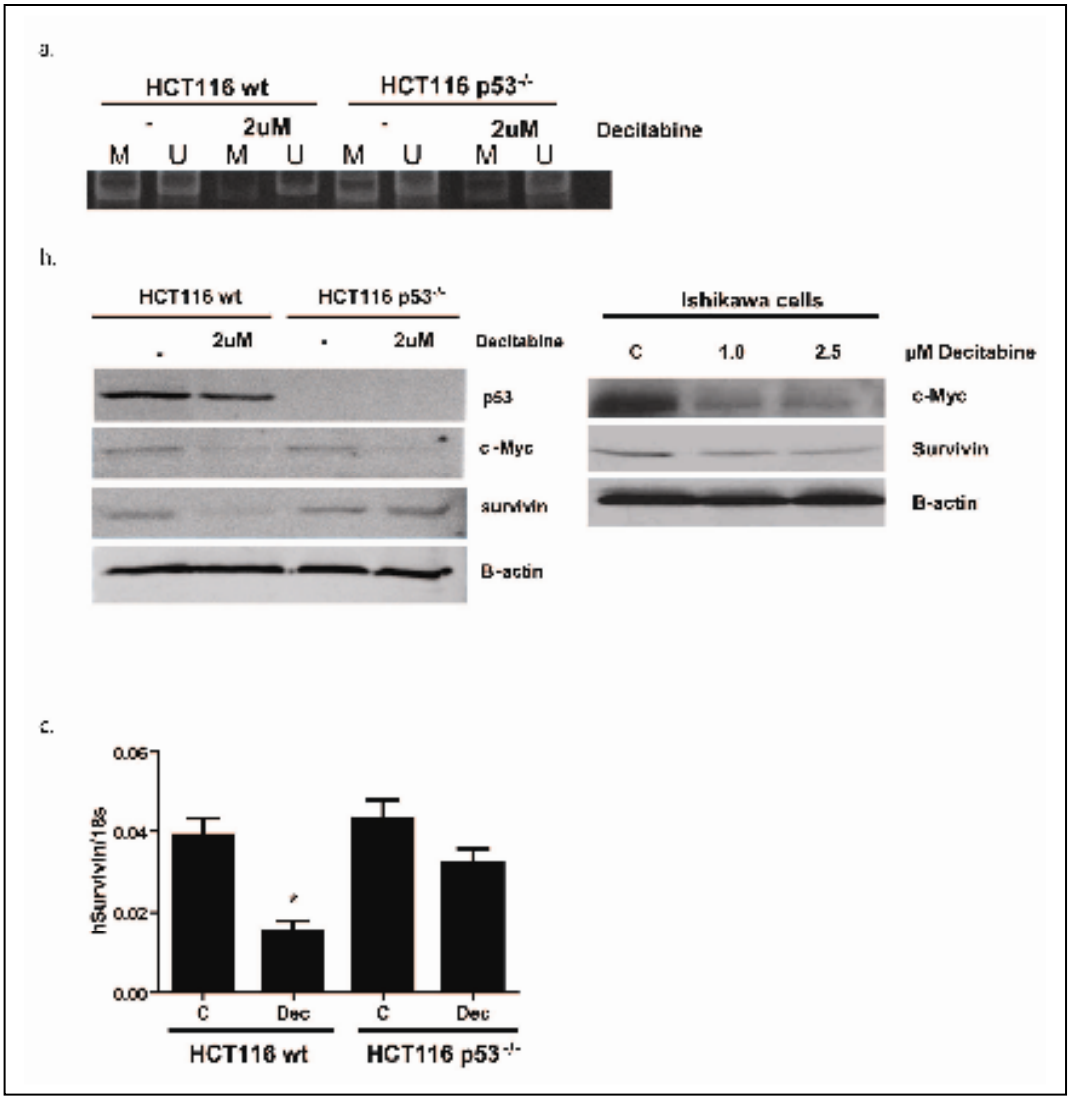

Figure 15: Survivin is repressed by decitabine in a p53 dependent manner

HCT116 and/or Ishikawa cells were treated with decitabine for 4 days then harvested for analysis. (a.) genomic DNA was isolated, bisulfite treated and incubated with MSP primers for methylated (M) or unmethylated (U) survivin DNA. (b.) Immunoblot analysis of isolated protein from all three cell lines. B-actin serves as a loading control, c-Myc is a biological control for drug induction. Band densities for survivin in the Ishikawa cells were caculated and normalized to B-actin band densities: C=0.57, 1μM=0.51 and 2.5μM=0.57 in relative units (c.) QPCR analysis of RNA isolated from HCT116 cells. $p<0.05$ by ANOVA followed by Tukey's ad hoc test

Modified from *Nabilsi et al Oncogene 2009*

Survivin repression by decitabine is not due to off-target high dose treatment

It has been observed that high dose decitabine treatment often causes non-specific off-target effects. To confirm that the survivin repression we observed was not due to off-target effects of high dose (2μM) drug treatment, we repeated the experiment with a low dose 200nM decitabine treatment. We again observed by QPCR significant survivin repression in the decitabine treated wild-type cells (58% repression, $p<0.05$) but not the p53-null cells (21%, non-significant) (Figure 16a). QPCR analysis of c-Myc gene expression served as a control for drug induction and we observed significant repression of c-Myc following decitabine treatment in both cell lines (Figure 16b). We concluded that the repression of survivin was not due to off-target high dose treatment.

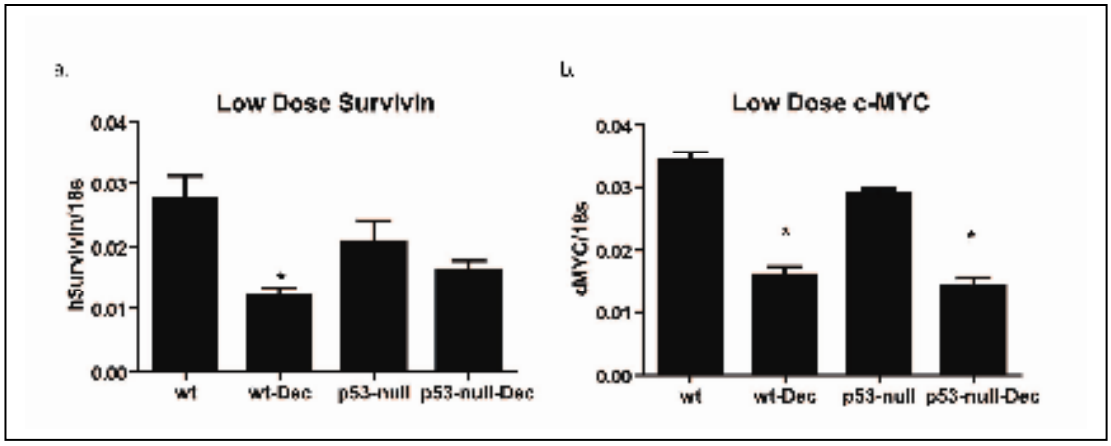

Figure 16: Low dose decitabine treatment of HCT116 cells

QPCR analysis of RNA isolated from HCT116 cells following 4 day treatment with low dose 200nM decitabine. (a.) Survivin levels and (b.) c-Myc levels are normalized to 18s. *$p<0.05$ by ANOVA followed by Tukey's ad hoc test.

Survivin repression by decitabine is not due to non-specific cell cycle effects

One important consideration in comparing the effects of decitabine on survivin expression in the wild-type and p53-null cells is the effect of decitabine on the cell cycle. Survivin expression is cell cycle regulated, increasing during G2 and M phases of the cell cycle. If the wild-type cells arrest in G1 whereas the p53-null cells arrest in G2 in response to drug treatment then this variation in cell cycle arrest could explain the observed survivin repression in wild-type cells but not p53-null cells.

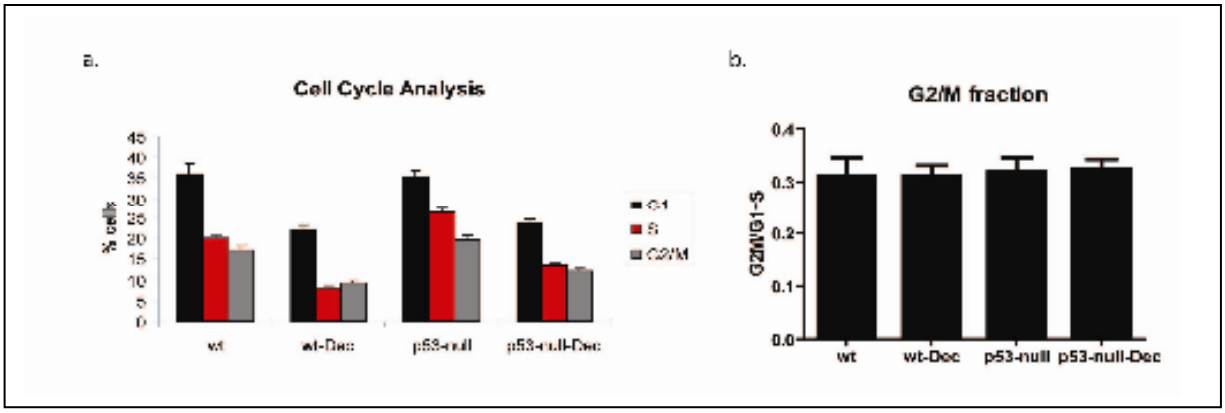

Figure 17: Cell cycle profile of decitabine treated HCT116 cells
Propidium iodide staining of HCT116 cells treated with decitabine (Dec) or vehicle indicated the DNA content and thus (a.) the percentage of cells in each stage of the cell cycle. (b.) Ratio of cells in the G2/M phase of the cell cycle.

To determine if survivin repression by decitabine is due to non-specific cell cycle effects, wild-type and p53-null HCT116 cells were treated with decitabine or vehicle for four days and then harvested for cell cycle analysis by propidium iodide staining. The mechanism of decitabine action suggested that the drug would cause both cell lines to arrest

in S-phase however cell cycle analysis indicated that both wild-type and p53-null cells mildly arrested in G1 phase (Figure 17a). To determine if there were any cell cycle changes that would variably affect survivin expression, we calculated the ratio of cells that were in G2/M-phase before and after treatment in both cell lines and determined that there was no difference in the G2/M phase fraction in the wild-type cells compared to the p53-null cells following decitabine treatment (mean ratio wt=0.31, wt + decitabine=0.31, p53-null=0.32, p53-null + decitabine=0.32; p=0.98 by ANOVA) (Figure 17b). We concluded that survivin repression by decitabine was not due to non-specific cell cycle effects.

Survivin repression by decitabine is not due to non-specific alterations in proliferation index

Survivin expression in many cases is associated with the proliferation index of the cell or tissue type in which it is measured and decitabine treatment often causes cells to slow or stop proliferating. Therefore it is important to determine if the observed repression of survivin in wild-type cells but not p53-null cells is due to a non-specific alteration of the proliferation status of the wild-type cells but not the p53-null cells in response to drug treatment. To determine the proliferation status of the cells, we measured the levels of Ki67, a nuclear antigen commonly used as a marker of proliferation, by QPCR. We found that there was a non-significant decrease in Ki67 transcript levels following drug treatment, however the decrease was similar in both cell lines (wt= 29% decrease, p53-null= 27% decrease) (Figure 18a) and could therefore not account for the 62% decrease in survivin levels in the wild-type cells compared to the 27% decrease in survivin levels in the p53-null cells (Figure 15c). Interestingly, the change in survivin and Ki67 levels was equal (27%) in

the p53-null cells suggesting that the observed small survivin repression in the p53-null cells was due to decreased cellular proliferation. To normalize for this proliferation effect, we calculated the ratio of survivin/Ki67 for each sample and found that normalizing for non-specific proliferation effects greatly augmented the observed p53 dependent survivin repression in response to decitabine treatment (Figure 18b). We concluded that the repression of survivin by decitabine in the wild-type cells was not due to a non-specific proliferation effect.

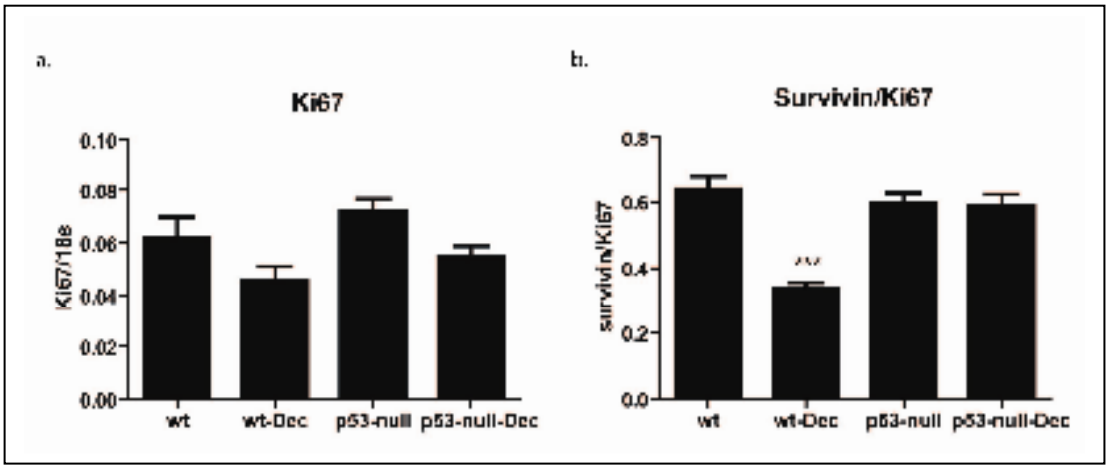

Figure 18: Proliferation index of HCT116 cells following decitabine treatment
QPCR analysis of RNA isolated from HCT116 cells following 4 day treatment with low dose 200nM decitabine. (a.) Ki67 levels are normalized to 18s (b.) survivin levels are normalized to Ki67. ***$p<0.0005$ by ANOVA followed by Tukey's ad hoc test.

Methylation of the survivin p53/E2F binding site inhibits p53 binding but not E2F1 binding *in vitro*

We have shown that demethylation of the survivin promoter results in survivin repression through a p53 dependent mechanism. Next we explored whether the methylation state of the survivin promoter could affect the DNA binding ability of p53. Methylated and unmethylated double stranded oligonucleotide probes representing the p53/E2F binding site sequence from the survivin promoter were end labeled for gel shift analysis. We observed a strong gel shift in the presence of purified human p53 protein with the unmethylated probe but a greatly diminished shift with the methylated probe (Figure 19a) indicating that p53 binding to the survivin promoter was reduced by DNA methylation. Pre-incubation with an anti-p53 antibody resulted in complete elimination of the gel shift signal indicating a specific interaction between the probe and p53.

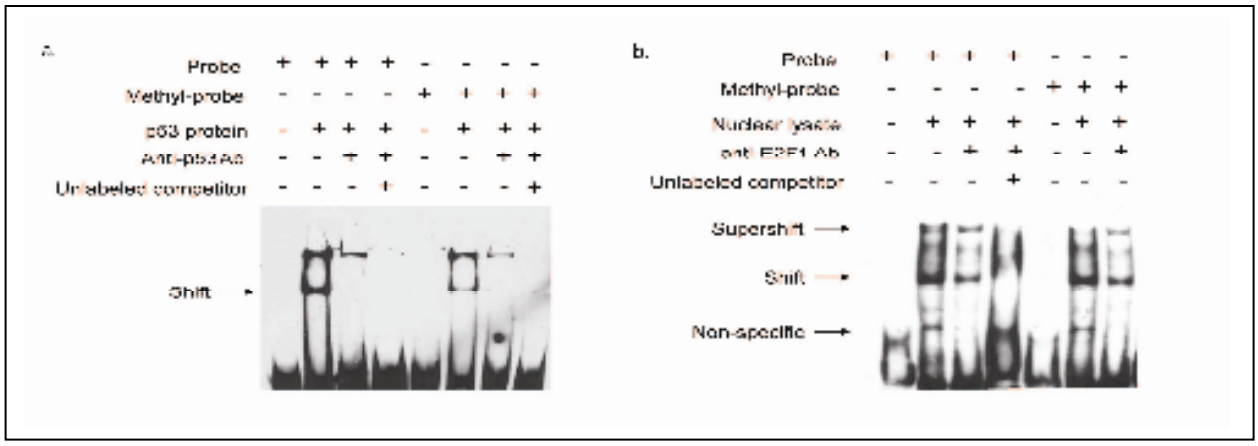

Figure 19: Gel-shift analysis of the survivin p53/E2F binding site

Gel shift assay of biotin labeled methylated or unmethylated probe incubated with (a.) purified p53 protein or (b.) E2F1 enriched nuclear lysates. Antibodies to p53 and E2F1 and unlabeled probe serve as controls for the specificity of the reactions.

Modified from *Nabilsi et al Oncogene 2009*

The transcription factor E2F1 is an activator of survivin and its binding site overlaps the p53 binding site in the survivin promoter. To determine if methylation specifically inhibits p53 binding or if E2F1 binding would be similarly affected, we incubated nuclear lysates enriched with E2F1 protein with the methylated and unmethylated probes. We observed that E2F1 binding resulted in an equal gel shift for both probes and was therefore not affected by methylation (Figure 19b). Pre-incubation with an anti-E2F1 antibody resulted in a diminished gel shift signal and the presence of a supershift indicating a specific interaction between the probe and E2F1. We concluded that DNA methylation can specifically inhibit the binding of p53 to the p53 binding site in the survivin promoter.

Demethylation of the survivin promoter increases p53 binding in HCT116 cells

To determine if DNA methylation affects p53 binding to the endogenous survivin promoter, we treated HCT116 wild-type cells with decitabine as described above to demethylate the survivin promoter and conducted chromatin immunoprecipitation (ChIP) analysis. Sheared chromatin from drug or vehicle treated cells was incubated with an anti p53-antibody to pull down DNA bound by p53. The DNA was purified and PCR amplified with survivin specific primers to determine the relative occupancy of p53 on the methylated (vehicle treated) vs. unmethylated (decitabine treated) survivin promoter. Anti-IgG antibodies were used in a parallel reaction to control for non-specific DNA pull-down. We observed a bright PCR product indicating enrichment of p53 occupancy on the survivin promoter following decitabine treatment compared to control and there was no band in the IgG negative control lane (Figure 20a). We quantified the band densities of PCR product from the pull down reactions by densitometry and normalized against the PCR products

from the input and observed a 4-fold increase in signal from the decitabine treated cells compared to control (Figure 20b). These results show that methylation can inhibit p53 binding to the endogenous survivin promoter in cells.

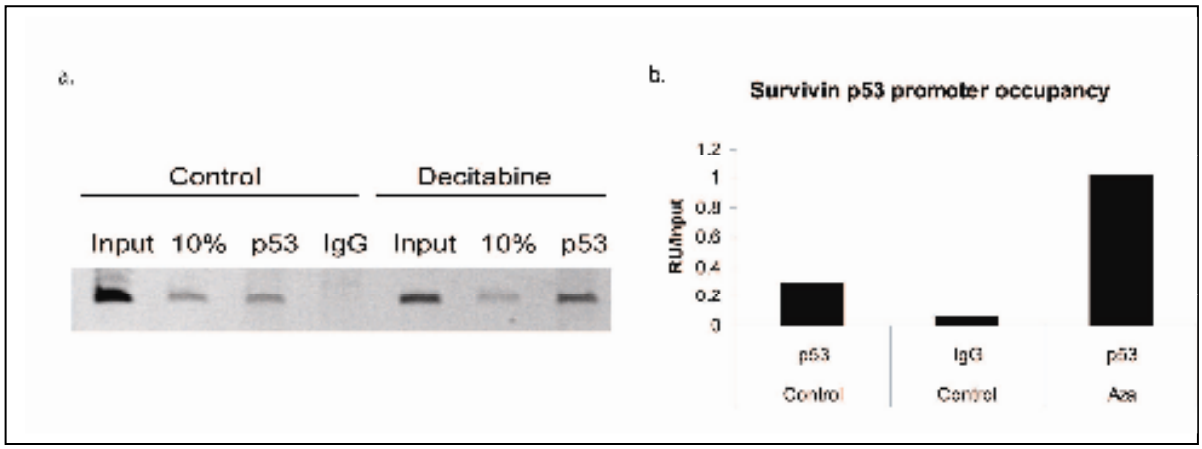

Figure 20: Chromatin immunoprecipitation analysis of the survivin promoter
Wild-type HCT116 cells treated with decitabine or vehicle for 4 days were harvested for ChIP analysis with anti-p53 and anti-IgG antibodies. Immunoprecipitated DNA was amplified with survivin specific PCR primers and (a.) PCR products were analyzed by PAGE. (b.) Band densities were quantified by densitometry.

Discussion

We have shown that survivin expression is regulated by DNA methylation and that demethylation of the survivin promoter by decitabine results in specific survivin transcript and protein repression through a p53 dependant mechanism. These results are significant because DNA methylation is understood to silence, not activate gene expression. This is largely because gene regulation by DNA methylation is considered to be an "all-or-none"

phenomenon whereby a gene is either silent or expressed and the silence is associated with nearly 100% promoter methylation. However physiologically, promoter methylation occurs at varying dosages not just 0% or 100%, indicating that there may be more to gene regulation by promoter methylation than just on-or-off. While the survivin promoter in human endometrial tumors was significantly hypermethylated compared to normal tissue, we never observed anywhere near 100% methylation of the survivin promoter in any of the tumors or cell lines, however with increasing methylation we observed increased survivin expression. Our results suggest that "low dose" promoter methylation may modulate gene expression and it is not necessarily an "all-or-none" event, nor is it restricted to gene silencing. While 100% promoter methylation will likely inhibit the basal transcriptional machinery from binding, lower doses of methylation may not be sufficient to interfere with the binding of such a large complex but it can interfere with other transcriptional regulators, including transcriptional repressors (which would result in gene activation).

We explored the mechanism of increased survivin expression by promoter methylation and found by gel shift and ChIP that p53 binding to the survivin promoter is specifically inhibited by DNA methylation. While there are several reports indicating that a link exists between p53 and DNA methylation (p53 represses DNMT1 (DNA methyl-transferase 1) expression and can scaffold DNMT1 protein binding to promoters), to our knowledge we are the first to report that DNA methylation can inhibit p53 from binding to a target gene's promoter. It is tempting to speculate that an auto-inhibitory mechanism exists whereby p53 binds to its target gene's promoter and recruits DNMT1 to reversibly methylate the promoter thus inhibiting its own future binding until either a de-methylation

signal is initiated or until it represses DNMT1 expression to the point that promoter methylation can not be maintained. Since aberrant DNMT1 overexpression is often observed in tumors, this DNMT1-p53 interaction may be augmented causing hypermethylation at p53 binding sites thus leading to aberrant inhibition of p53 binding and action. In the next chapter we will further explore the relationship between promoter de-methylation and gene expression.

CHAPTER 5: MICROARRAY ANALYSIS IDENTIFIES SEVERAL GENES THAT ARE REPRESSED BY DECITABINE TREATMENT

Introduction

One experiment that is commonly used to determine if a candidate gene is regulated by DNA methylation is to treat cells with decitabine and observe the effect on the expression levels of the gene in question. Since these studies are often conducted under the assumption that methylation only silences gene expression, data analysis is focused on genes that are activated by decitabine treatment, not genes which are repressed. We have reported data that shows activation of survivin expression by promoter methylation. Next we wanted to determine if there are other genes that are also activated by DNA methylation. We therefore treated Ishikawa and HCT116 cells with decitabine then conducted microarray analysis to measure changes in gene expression but focused our attention on genes that are repressed, not activated by decitabine treatment. In this chapter we will report genes that are repressed by decitabine in Ishikawa cells, and genes that are repressed in HCT116 cells in a p53-dependent manner.

Materials and Methods

Microarray Analysis Using BeadChip Arrays

Microarray experiments were conducted on two independent RNA samples from HCT116 cells and Ishikawa cells. RNA was isolated as described above and microarray analysis was conducted using HumanRef-8 BeadChip arrays from Illumina. RNA was amplified and hybridized according to the manufacturer's protocol. Briefly, RNA is amplified and then cDNA synthesized via reverse transcription. The cDNA is converted to cRNA containing biotinylated UTPs and then incubated with avidin labeled Cy3 dye. Each

sample was then added to a separate array on the bead chip and incubated in a warm rotisserie overnight. Next day, bead chips are washed and scanned. Following scanning, Bead Studio 3 (Illumina) software was used for data analysis. Bioinformatic pathway analysis was conducted utilizing Ingenuity software.

QPCR analysis was conducted to validate microarray results. Assays were conducted as described in chapter 2 with the exception that UPL (universal probe library) probes were used in place of Taqman probes and analyses were conducted using the ddC_T method (as described for the CNV assay in chapter 2) without a standard curve.

Statistical Analysis

Differences for QPCR validation of microarray results were calculated by unpaired t-tests. Differences were considered significant if $p<0.05$. For microarray analysis, after background subtraction arrays were normalized to each other by quantile normalization. Changes in gene expression were tested using a modified t-test that employs estimates of variation that include sequence specific biological variation (s_{bio}), nonspecific biological variation (s_{neg}) and technical error (s_{tech}) (Illumina User Guide, rev B. page 6-11 – 6-12, 2005, Illumina Inc) . Genes were considered differentially regulated at $p<0.001$.

Results

Microarray analysis identifies genes that are repressed by decitabine in Ishikawa cells

Expression analysis of RNA isolated from Ishikawa cells grown in 2uM decitabine for 4 days (all in duplicate) showed that of the 22,000 transcripts present on the chip, after quantile normalization, 2,752 were changed with a p-value ≤ 0.001. Scatter plot analysis indicates that of the 2,752 changed genes, 1,261 genes showed increased expression indicating activation by demethylation (the traditional view of decitabine action) and 1,429 genes showed decreased expression indicating repression by demethylation (Figure 21c). The scatter plot analysis also shows that the gene expression signatures of the biological duplicates for control and drug treated cells were incredibly similar with r^2 values of 0.99 and 0.98 respectively indicating positive quality control (Figure 21a. and b.).

Bioinformatic pathway analysis of the results indicated that several of the repressed genes are involved in cell cycle regulation and are targets of p53 (Table 2) indicating that this mechanism of repression by demethylation occurs in genes other than survivin and in a different cell-type. Notably, survivin expression was not changed following decitabine treatment in these cells consistent with our findings that the survivin promoter is unmethylated in Ishikawa cells and that survivin protein levels were not changed in these cells following drug treatment (Chapter 4). However, c-Myc levels were reduced by 51% again consistent with the immunoblot data we showed previously.

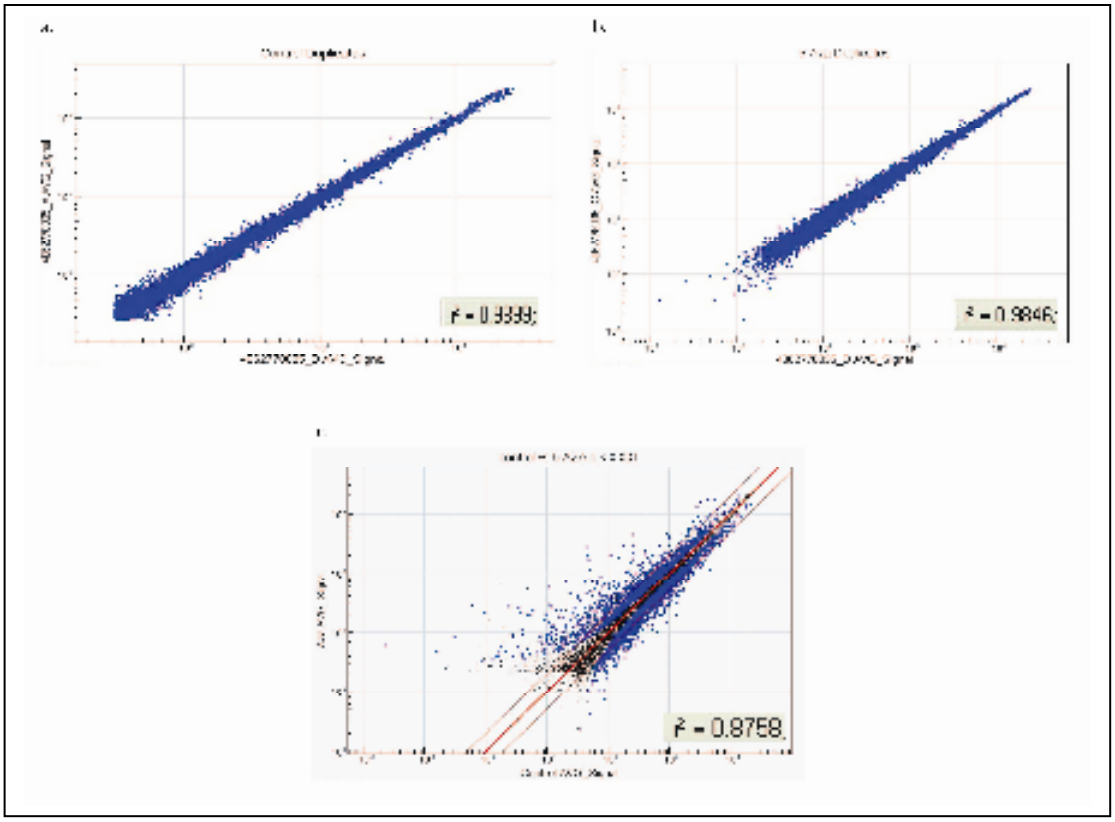

Figure 21: Microarray scatterplots of decitabine treated Ishikawa cells
(a.) Comparison of two control cell samples, r^2=0.99 and (b.) Comparison of two drug treated samples, r^2=0.98 indicating similar gene expression signatures of the biological controls. Blue marks indicate significantly expressed genes (c.) Comparison of control vs. drug treated, r^2=0.88 with gene changes of $p<0.001$ indicated in blue, red lines indicate the 2-fold change boundaries.

	Control	Decitabine	p-value
MYC	1263.1	670.9	6.87E-20
HK2	460.4	217.5	3.21E-19
CCNA2	2102	1148.9	6.83E-12
MAD2L1	2282	1474.5	1.02E-10
BAX	139.2	66.1	4.71E-09
DUT	926.6	578.9	5.76E-09
UBE2C	153.8	74.7	5.94E-09
CDC25C	385.5	247.7	1.06E-08
TMEM97	3900	2190.3	7.12E-08
CRIP2	1939.3	1361	2.20E-07
PSRC1	952.4	700.4	4.96E-07
RFC3	77	23	1.11E-06
CDK4	3783	2455	1.13E-06
PBK	1169.6	711.9	1.71E-06
CENPF	1144.4	718.5	3.18E-06
CCNG1	1802.1	1046.9	3.54E-06
TOP2A	3395.1	2659.6	4.14E-05
BUB1B	1027.3	796.2	4.57E-05
AURKB	1196.5	767.4	1.71E-04
PODXL	171.7	83.9	2.72E-04
RAC1	398.1	276.4	8.60E-04

Table 2: p53 regulated genes repressed by decitabine treatment in Ishikawa cells

Gene symbols, signal and p-values are indicated for control and drug treated cells ascending by p-value. Genes in bold are implicated in cell cycle control.

Microarray analysis identifies 42 genes which are repressed by decitabine in a p53 dependant manner

To determine if other genes are de-repressed by DNA methylation by a p53 mediated mechanism, we obtained RNA from HCT116 wild type and p53-null cells treated with decitabine or vehicle (all in duplicate) then analyzed gene expression by microarray. We observed that 325 genes were repressed more than 2-fold (3,056 total), $p<0.001$ by drug

treatment in the wild type cells and 251 genes were repressed more than 2-fold (2,559 total), $p<0.001$ in the p53 null cells. To confirm that the drug treatment reproduced results reported by others, we examined the expression status of 5 genes (*NKX2-5, SPOCK2, SLC16A12, DPY5,* and *GALR2*) reported to be epigenetic biomarkers of colon cancer in human tumors and that are re-expressed following decitabine treatment in colon cancer cell lines (16). We found that 3 of the 5 genes (*NKX2*, *SPOCK2*, and *GALR2*) were re-expressed following decitabine treatment in both cell lines whereas the other 2 did not change. Since *GALR2* is the most predictive of the 5 genes (85% sensitivity, 95% specificity for predicting colon cancer), we were confident that decitabine treatment was successfully administered in these cells.

We ranked the genes repressed in the wild type cells by p-value then compared those numbers to the p-values of the same genes in the p53 null cells. We identified 50 genes which exhibited a statistically significant repression following decitabine treatment in the wild-type cells but a non-significant (or less significant) change in the p53-null cells. Subsequent analysis indicated that 46 out of the 50 identified genes (92%) contained canonical CpG islands in their promoters and/or within their first 2 exons. These 46 genes are listed in Table 3. Notably, the majority of these genes are associated with cancer progression and/or cell proliferation.

We selected a subset of genes: *HMGB1, UNC84b, Nek2, CDC25C* and *CCNF* for further validation. QPCR analysis was conducted on an independent set of control and treated cells from both cell lines. Validation studies indicated that *HMGB1, Nek2* and *CDC25C* all exhibited statistically significant, p53 dependent, gene repression by decitabine

treatment (*HMGB1* mean wt repression = 65%, mean p53null repression = 30%, p=0.042; *Nek2* wt = 68%, p53null= 10%, p=0.0088; *CDC25C* wt = 89%, p53null = 59%, p=0.0099) (Figure 22). QPCR analysis indicated that UNC84b and CCNF were also repressed by decitabine treatment, however by QPCR, this repression was not dependent on p53 (data not shown).

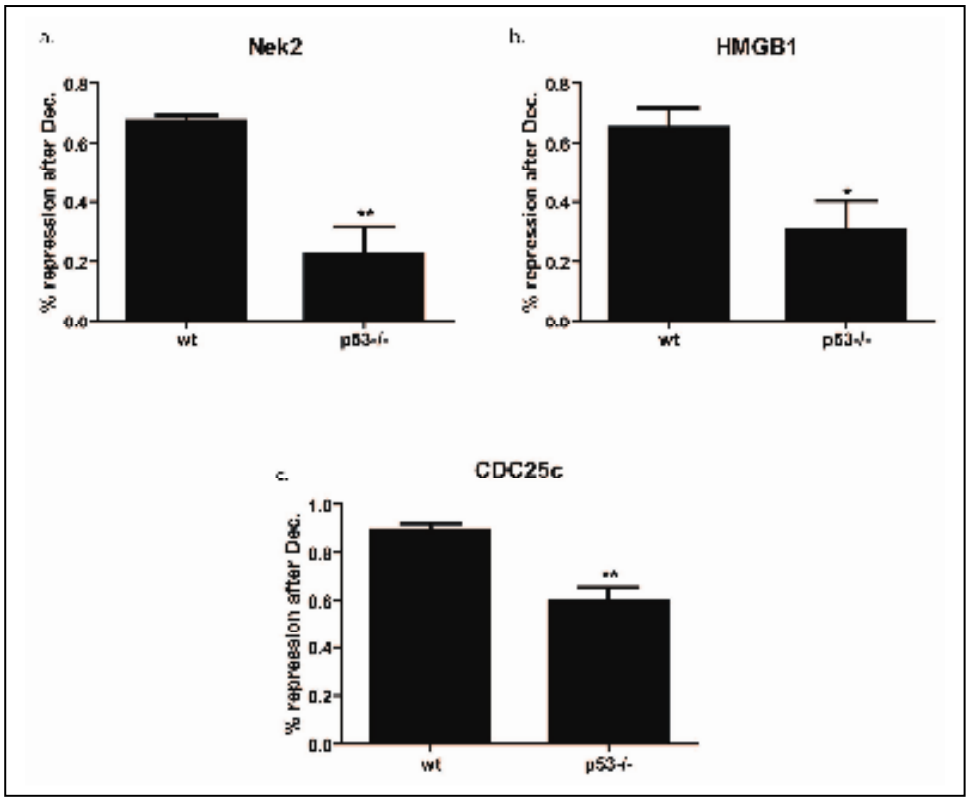

Figure 22: Validation studies of microarray results

QPCR assays for (a.) Nek2 (b.) HMGB1 and (c.) CDC25C were designed and transcripts measured in RNA isolated from an independent treatment experiment. Data were quantified using the ddC_T method and are presented as %repression compared to control for each cell type. *p<0.05, **p<0.005, unpaired t-test, n=3.

Data in *Nabilsi et al, Oncogene 2009*

Symbol	wt	wt+dec	p-value	p53null	p53null+dec	p-value
Cell cycle						
NEK2	488.4	243.8	7.36E-38	480.9	462.3	1.65E-01
AMH	86.9	39.6	1.77E-05	101.8	76	3.15E-01
HD	65.1	27.4	8.67E-04	93.1	55.2	5.30E-01
GLI2	90.2	43.4	2.36E-05	91.2	58.4	1.05E-03
NEDD4L	174.3	69.6	6.52E-18	161.1	111.8	1.15E-03
NUMA1	71.1	33.8	5.75E-04	72.6	38.5	2.39E-03
UNC84B	185.6	86.1	6.06E-16	140.1	140.1	4.80E-03
TNKS1BP1	152.4	72.4	4.74E-08	163.3	125.1	1.46E-01
FAM29A	92.3	39.9	1.75E-04	101.8	60.2	1.64E-02
CCDC18	126.3	55.6	1.52E-06	124.9	87.8	7.87E-03
CDC25C	331.2	158	3.89E-32	341	241	4.97E-09
CENPF	1066.4	441.8	6.26E-27	1152.7	600.9	1.12E-14
CEP110	161	63	5.99E-10	123.8	81.8	3.01E-07
CEP57	126.5	59	4.31E-09	93.9	70.5	3.49E-06
CCNF	395	192.6	2.40E-37	339	319.1	6.20E-06
Cell death						
HMGB1	339.3	168.6	7.44E-12	294.1	357.2	7.50E-01
PTPN9	62.8	31.1	4.57E-04	66.3	45.1	1.29E-02
NRTN	81.2	36.4	4.40E-05	82.2	52.9	3.58E-03
MYO18A	127.9	57.1	3.15E-04	128.9	85.5	9.24E-02
RTN3	324.8	160.1	2.61E-13	338.5	243.1	2.46E-03
BAG2	91.5	41.3	8.94E-04	91.2	68.5	1.45E-01
Development						
CAPN5	74.7	23.1	4.00E-04	73.3	48.1	1.32E-01
FZD7	93	43.5	8.20E-06	67.5	65.4	4.53E-03
BSN	51	9.4	9.38E-05	58.4	21.5	1.24E-03
RAPGEFL1	84.9	35.5	1.25E-05	52.3	56	4.33E-02
LMNB1	879.1	431	4.79E-04	803.6	443.1	1.38E-02
NR2F1	755.9	282.2	7.36E-38	310.9	220.3	7.36E-38
Cancer						
MSI1	71.1	18.9	2.61E-06	92.1	46.7	7.13E-02
RPSA	255.1	115.7	1.11E-11	332.9	214	9.79E-02
TMEM18	71.1	27.6	6.05E-05	89.2	32.8	6.39E-03
MET	1442.9	541.2	7.36E-38	497.5	323.5	7.36E-38
Nucleic Acid catabolism/metabolism						
OLA1	53.1	15.7	4.55E-04	55.8	35.2	3.68E-02
ALDH6A1	290.8	142.1	1.91E-26	393.6	236	6.99E-03
PABPC3	287.1	129	4.49E-27	299.1	247.5	1.26E-02
DNA binding						
ENOX1	44.6	6.7	3.42E-04	47	15.2	1.39E-03
ZC3H3	164.4	79	3.27E-06	189	105	1.08E-03
NFIX	832.2	378.6	7.36E-38	499.4	401.5	7.36E-38
Unknown/Miscellaneous						
SH3RF2	92.6	45.7	2.33E-05	156.1	105.5	2.26E-01
NXPH4	72.1	35.3	3.83E-04	88.9	55	2.20E-01
LZTFL1	214.2	100.2	4.15E-17	207.3	153.9	5.06E-02
C4ORF29	67.7	29.4	4.05E-04	47.9	33.2	8.25E-03
TBC1D17	91.1	42.8	4.15E-04	68.1	61.5	3.13E-02
REEP3	61	18.2	7.11E-05	73.8	33.7	5.56E-03
GPR162	89.4	38.5	1.35E-08	111.6	62.8	2.52E-03
C3ORF62	65.1	20.9	9.50E-04	43.4	26.5	2.18E-03
SAMD5	87.1	25.7	7.51E-08	60.4	50.1	1.01E-03

Table 3: Genes repressed by decitabine in a p53 dependant manner

This table lists genes that are significantly changed in the wild type cells but not in the p53 null cells. Genes are grouped by function from Ingenuity software analysis

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Discussion

In this chapter we conducted microarray experiments and uncovered several genes that are repressed following decitabine treatment. Conducting these experiments in HCT116 cells allowed us to uncover 46 genes that may be regulated by methylation through a p53-dependent mechanism. Further studies of the methylation status of these candidate genes will be necessary to determine if they are truly repressed by demethylation. We concluded that gene de-repression by methylation may be a common mechanism of gene regulation that has been previously unrecognized. There were also several genes that were repressed by decitabine treatment in a p53 **independent** manner indicating that other transcriptional repressors may be regulated similarly.

We also observed that an interesting set of genes that were completely unexpressed in the control cells then became highly expressed (up to 10,000-fold) following decitabine treatment in both HCT116 cell types. If we express the data as a scatter plot, we can see that these genes cause a "spur" on the left-hand side of the plots (Figure 23 a, green bracket). Many of these genes are members of a G-antigen protein family (GAGE). The GAGE family transcripts and proteins are cancer/testis specific antigens meaning they are highly

expressed in primordial male germ cells during development and are often expressed in different types of cancers. Intriguingly, most of these testis-specific GAGE genes are located on the X-chromosome. The actual function of these proteins remains unknown however they are potent activators of T-cell responses and are currently being studied as targets for cancer immunotherapy. Their robust activation by decitabine in HCT116 cells suggests that they are silenced by methylation in this cell type. Perhaps coupling decitabine treatment with T-cell based immunotherapy may enhance the efficacy of immune-based treatments in patients with lower GAGE presentation?

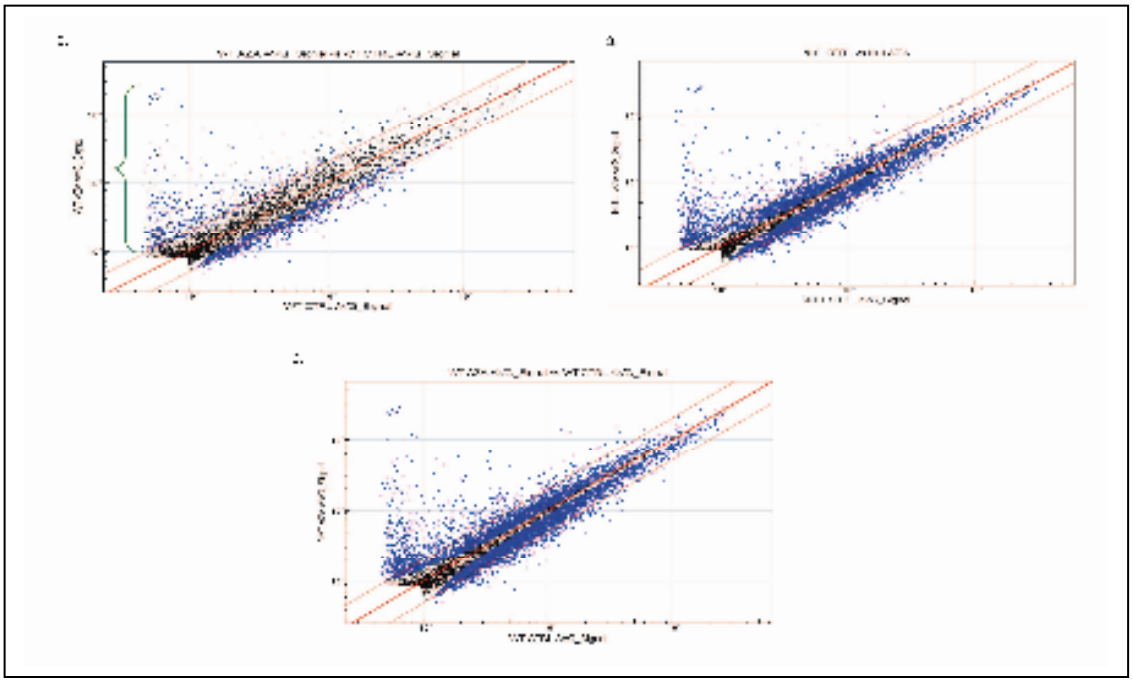

Figure 23: Microarray scatter plots of decitabine treated HCT116 cells
Data following quantile normalization from (a.) wild-type cells control vs. dec., n=2 (b.) p53-null cells control vs. dec., n=2 and (c.) wild-type cells control vs. dec. Blue dotes represent genes changed by $p<0.001$, red lines indicate the 2-fold range boundaries and the green bracket highlights the "spur" of genes that are silent and become highly expressed following drug treatment

The scatter plots also suggest that more genes were changed following decitabine treatment in the p53-null cells than in the wild-type cells (Figure 23 a. vs. b.). This is actually due to high variability between decitabine wild-type replicates rather than a biological reality. One of the treated wild-type samples changed much more radically than its biological replicate thus confounding the statistics and dampening the statistical significance of the changes. If we remove this sample from the analysis and re-analyze the data with n=1 for this group, we observe very similar numbers for genes changed following treatment in the wild type cells and in the p53-null cells (Figure 23c). To maintain biological relevance, we conducted our analyses with the smaller set of genes from the n=2 sample set. This greatly diminishes the number of candidate genes that we have to work with however it also decreases the number of false positives and is therefore more likely to be biologically relevant.

Finally, we compared the gene lists for expression changes of at least 2-fold repression with $p<0.001$ from all three cell types following decitabine treatment. We found that 525 genes are similarly repressed in the HCT116 cells but there are genes that change specifically for each cell type (300 in wild-type, 209 in p53-null), indicating that theses genes may also be regulated by p53 or they may have a different methylation status in each cell line (Figure 24). Two hundred and ninety-nine genes changed specifically in Ishikawa cells again suggesting that their methylation status in these cells differs from the HCT116 cells. There are also 40 genes that are reliably repressed in all 3 cell types. These 40 genes that are common to all groups are listed in Table 4. These genes are either consistently methylated in all tested cell lines and are repressed by demethylation like survivin, or they

represent non-specific off-target effects of drug treatment that can not be attributed to their methylation status. Thirty-three of the 40 genes (83%) contain canonical CpG islands in or near their promoter regions. This enrichment of CpG islands suggests that they are likely exhibiting methylation-based repression rather than non-specific effects. Further studies exploring the methylation status of these candidate genes will be necessary to determine whether their repression by decitabine treatment is due to regulation by DNA methylation.

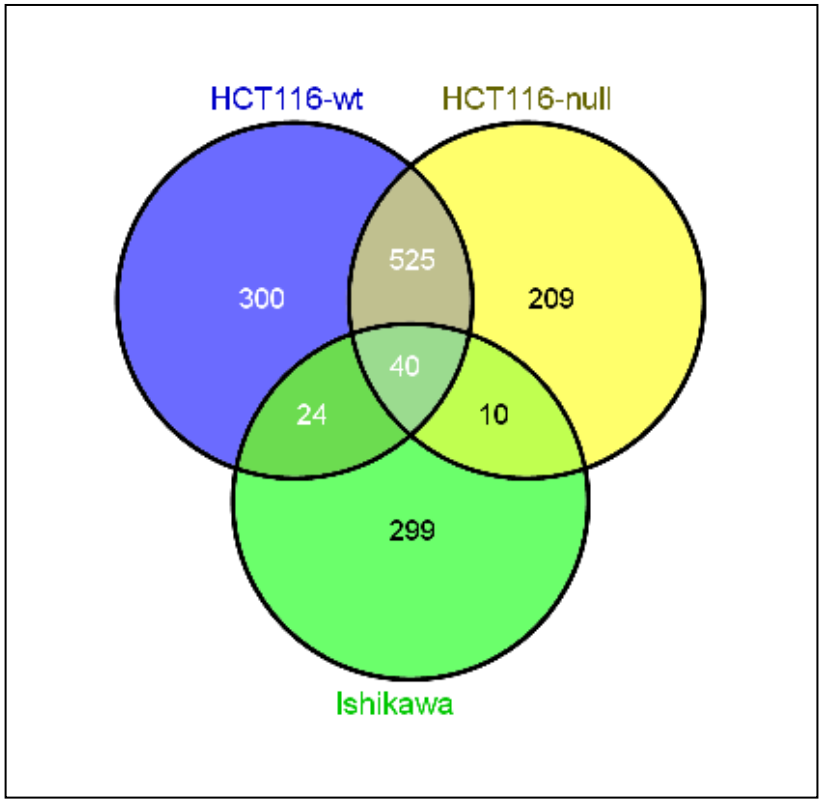

Figure 24: Venn diagram of microarray results

Comparison of genes that were repressed more than 2-fold by decitabine treatment. Overlaps indicate shared genes for the indicated cell type.

Gene	Island size (bp)	Ratio	Location
ACOX2			
ACSM3			
ACY1L2	893	0.93	promoter
ALDH3A2	1139	0.75	promoter
ARHGAP18			
C14orf93	222	1.06	promoter
	505	1.02	intron 1
DDIT4	1531	0.75	promoter-exon3
ESPN	824	1.03	promoter, 5 more islands throughout
EVA1			
FABP6			
FECH	549	0.94	promoter
GALNTL4	1698	0.81	promoter
GBAS	857	1.02	promoter
HNRPDL	2406	0.93	promoter-intron1
LHPP	497	1.05	promoter, 2 more throughout
LRRC20	1077	0.84	promoter
LRRC45	1349	0.79	promoter, 3 more throughout
MRPL11			
MYB	2257	0.95	promoter-intron1
NFIA	811	1.12	promoter
NIBP	299	0.79	promoter, 5 more throughout
NICN1	365	0.85	promoter
NSBP1			
NTHL1	900	0.9	promoter-intron1
OSGEPL1			
PFKFB4	831	0.66	promoter
PPA2	771	0.87	promoter
RAMP1	758	0.99	promoter-intron1
SCD	1540	0.82	promoter-intron1
SCNN1A	662	0.74	exon2
SLC16A14	1082	0.79	promoter-intron1
SLC29A2	923	0.8	promoter-exon2
SREBF1	1063	0.91	promoter-intron1, 2more throughout
SRI	591	0.86	promoter-intron1
THNSL1	706	0.86	promoter-intron1
TJP3	354	0.77	exon4
	259	0.91	exon18
TLOC1	612	0.88	promoter-intron1
WDR4	697	0.93	promoter
YEATS4	643	1.01	promoter-intron1
ZNF695	566	0.78	promoter-intron1

Table 4: Genes repressed at least 2-fold in all cell types

Gene symbols for 40 genes repressed by decitabine in all cell types are listed along with the size of their corresponding CpG islands, the ratio of observed/expected CpGs (must be greater than 0.6 to be considered an island) and the location of the island on the gene. Blank CpG island information indicates lack of a CpG island for that gene.

CHAPTER 6: SUMMARY, SIGNIFICANCE, FUTURE DIRECTIONS AND REFERENCES

Summary and Significance

Over the past 2 decades there has been an exponential increase in the study of DNA methylation and its effects on human disease, especially cancer. Many dogmatic tenants have been challenged, such as the ideas that DNA methylation is irreversible and only functions to silence gene expression (19, 97). There are however many basic questions that have remained unanswered. What is the function of DNA methylation? What is the signal that marks a DNA sequence for methylation? What comes first, the silencing or the methylation? To begin to answer these questions we need to more fully understand the effect of DNA methylation on gene expression and the nuances of the interactions between methylated DNA and transcription factors.

Traditionally DNA methylation in mammals has been associated with gene silencing. This is because early studies of DNA methylation were focused on development and DNA methylation is the major mechanism for silencing gene expression during developmental imprinting and for X-chromosome inactivation in females (70, 95, 96, 107). More recently however, large scale genome-wide studies of DNA methylation patterns indicate that DNA methylation occurs more frequently on genes that are actively transcribed, in intergenic regions more than promoter regions (46), and that the active X-chromosome is more heavily methylated than the inactive X-chromosome (41). Furthermore, genome wide ChIP studies indicated that the methyl binding protein MeCP2 was more commonly found bound to genes which were actively transcribed than genes that were silenced (116). These results indicate

that we are just at the beginning of understanding the role of DNA methylation in regulating gene expression.

Our studies have uncovered two novel findings for epigenetic regulation by DNA methylation. One is that DNA methylation can activate gene expression of the oncogene survivin. The other is that the transcription factor p53 can be inhibited from binding to methylated DNA. We have shown that in a human disease, endometrial cancer, DNA methylation at a specific regulatory region of the survivin promoter (the p53 binding site) correlates with increased survivin expression. We have also shown that manipulation of the methylation status of the survivin promoter alters its gene expression and the ability of p53 to bind to the promoter. These results are important because they challenge the current understanding that DNA methylation only silences gene expression and that only the binding of methyl binding proteins are affected by DNA methylation. Furthermore, through microarray studies we have identified 46 candidate genes which may be similarly regulated indicating that this is an underappreciated mechanism of epigenetic regulation.

Clinically this project is significant because it may result in unveiling novel targets for epigenetically based cancer therapeutics. There may be undiscovered methyl binding factors involved in gene activation as opposed to gene silencing which could be targeted by pharmacologic inhibitors. Alternatively, decitabine is currently used mainly to treat leukemia. It is possible that this drug may be useful in treating solid tumors which retain wild type p53 expression. De-methylation could allow p53 to re-establish its function as a

tumor suppressor in these tumors. Our study would serve as a proof of principle to begin using decitabine to treat patients with these types of tumors.

Future Directions & References

There are several exciting directions that can be taken to further explore the novel findings uncovered in our study. Each candidate gene identified by microarray can be validated as a novel target of epigenetic and/or p53 regulation. We are currently conducting these types of studies on the mitotic kinase Nek2 which is an exciting target because it is overexpressed in several tumor types and is a biomarker of poor prognosis in breast cancer.

It would also be interesting to explore more fully the effect of DNA methylation on transcription factor binding. We showed by ChIP that if we de-methylate the survivin promoter by treating with decitabine that there is increased binding of p53 to the survivin promoter. We could expand this analysis by applying the DNA pulled-down by p53 (in pre and post treatment samples) to a Promoter Chip and look for genome wide promoter changes in p53 binding patterns in response to decitabine. This can then be expanded to include other transcription factors and we could then comprehensively describe the promoters and transcription factors that are affected by DNA methylation. This would not only expand our knowledge about the interaction between transcription factors and DNA methylation but may also help us uncover the true physiological function of DNA methylation by indicating the promoters and transcription factors with which it specifically interacts.

I would also be interested in determining which methyl binding proteins are recruited to the methylated survivin promoter and to determine if binding of these specific proteins is associated with gene activation as opposed to gene silencing. I would start with MeCP2, which has already been shown to preferentially bind to active genes in neuronal cells. If so, pharmacologic inhibitors of these methyl binding proteins may be developed as novel epigenetic cancer therapeutics. Along the same line of inquiry, I would like to determine how the histone modification signature surrounding the methylated survivin promoter compares to the histone signature of the unmethylated survivin promoter. There may be a novel histone marker for methylation activated genes compared to methylation silenced genes which could then be targeted with the many histone modification-based cancer therapies.

Our study could also lead to translational applications. We are currently collaborating with Dr. Peter Laird, the director of the Epigenome Center in California to explore bioinformatically if the genes which were identified as repressed by decitabine treatment in our microarray are commonly found to be methylated in their database of genome-wide methylation patterns in human tumors. If so, they would represent more cases of gene activation by DNA methylation and may persuade more laboratories to explore this possibility in their studies. They may also represent important targets for methylation based cancer therapy and may enhance the case for using agents like decitabine to treat solid tumors.

An alternate route to translating this work clinically would be to determine if this regulation of survivin by DNA methylation is tissue specific or if it also occurs in gliomas. There is a laboratory in Chicago, IL that has designed a viral therapy for malignant glioma whereby the survivin promoter drives the expression of an oncolytic virus (101, 102). If DNA methylation increases survivin expression in gliomas then they may be able to enhance the expression of their virus by simply methylating the survivin promoter *in vitro* prior to treatment.

In conclusion, I believe that we have made a strong case for the possibility that gene regulation by DNA methylation is more sophisticated than just an "on-off" phenomenon and that gene activation by DNA methylation may have biological consequences, including cancer progression. There may be more subtle changes in gene expression that are directed by or result from DNA methylation. Perhaps we should move away from the "on-off" toggle switch mentality and begin examining DNA methylation as a dial dimming/brightening switch?

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