SURVIVIN AND ITS EXPRESSION-CORRELATED GENES: A POSSIBLE REGULATORY MECHANISM INVOLVED IN COLON CANCER DEVELOPMENT

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Survivin (*BIRC5*), a member of the inhibitor of apoptosis family of proteins (IAP) is a 16.5kDa protein that is highly over-expressed in many types of cancer including colorectal cancer (CRC), but is almost undetectable in normal tissue. It has been considered a biomarker for tumor progression. Over-expression of survivin predicts poor patient survival and high tumor recurrence rates (Sarela, Macadam et al. 2000).

Preliminary large scale expresseion analysis between healthy and diseased colon revealed that most of the genes that were expression-correlated with survivin were classified as cell-cycle and mitosis genes. Survivin itself is expressed preferentially during mitosis and is needed for spindle assembly checkpoint (Mita, Mita et al. 2008).

Survivin is known to interact with a high number of signaling molecules, regulators, transcriptional networks that, are involved in its functions directly or indirectly (Altieri 2008). To appreciate the complexity it is not enough to think of survivin alone, but it becomes apparent by performing network analyses that link survivin to multiple signaling circuits. This led us to predict that there may be many genes that are simultaneously regulated with survivin by a network of transcriptional regulators.

As the expression correlation analysis showed that there is a specific cohort of genes that are expression-correlated with survivin, I proposed that the expression of this set of genes can be co-regulated by a common set of transcription factors (TFs). To identify enriched regulatory elements supporting my hypothesis of co-regulation, the promoter regions of these genes were analyzed to identify shared transcriptional regulatory elements (TREs), which provide information on cognate TFs that may be involved in this co-regulation. Eleven TFs were selected corresponding to those TREs

that showed statistically significant over-representation in the set of expression-correlated genes. The panel included *TP53, MYB, E2F4, E2F1, PAX6, NKX2-5, USF1, NFE2, NFY-A, ELK1* and *TDP2*.

Nothing much is known about the TFs that co-regulate survivin and its group of expression-correlated gene especially in CRC. This led to my hypothesis *that specific TFs corresponding to common TREs in promoter regions of survivin and its expression-correlated genes are differentially expressed in normal versus malignant colon tissues at the mRNA as well as the protein level.*

For this thesis research project, differential expression of the candidate TFs was first investigated at the mRNA level using quantitative real-time polymerase chain reaction (Q-RT-PCR), to analyze the mRNA levels between human normal and tumor colon tissues. The results demonstrate that there is differential expression of mRNA between CRC versus normal colonic epithelium in most of the TFs analyzed. Many of the TFs showed increased expression, which was statistically significant, in tumor tissue compared to adjacent normal colonic epithelium. To analyze whether changes at the mRNA level correspond to changes at the translational level, protein levels were analyzed using immunohistochemistry. Among the six Q-RT-PCR validated TFs tested by immunostaining, five (*TP53*, *MYB*, *E2F4*, *PAX6* and *NKX2-5*) showed differential expression in tumor compared to the normal colonic tissue. All these TFs showed increased expression in tumor tissue compared to normal except *E2F1*, which showed no change in expression in normal compared to tumor tissue consistent with mRNA quantification results.

In conclusion, my results indicated that out of the ten TFs tested for, more than six TFs showed increased expression in colon cancer tissue compared to normal colonic epithelium in mRNA as well as protein level. Even though, *NKX2-5* showed

increased expression of protein in tumor tissue compared to the normal colonic epithelium at the protein level, its mRNA levels showed negligible change between normal and tumor. *E2F1* showed no change in expression at either the mRNA or the protein level.

Survivin has been known to be over-expressed in many cancers including CRC (Altieri 2003). By large scale network analysis, it was shown that along with survivin many other genes are expression-correlated. My results indicate that TFs involved in the regulation of survivin and its expression-correlated genes that showed enrichment of TREs using computational analysis tool promoter analysis and interaction network toolset (PAINT), shows an increased expression in tumor tissue compared to the normal colonic mucosa in the mRNA as well as the protein level. This shows that cancer has a dysregulated transcriptional network, which might play a part in the dysregulation of survivin and its expression-correlated genes. Consequently this change in expression pattern of transcriptional regulatory molecules might play a role in changing the expression of survivin and its expression-correlated genes which may contribute to the progression and development of CRC.

These results might be used towards the development of a diagnostic test or a survivin cancer network-targeted antagonist based on the expression pattern and transcriptional regulatory network involved with survivin and its expression-correlated genes. Targeting survivin and its expression-correlated genes can be considered as a step in that direction with a CRC perspective. The study of survivin and its expression-correlated genes can be considered an invaluable study that sheds light on many pathways that are involved in CRC which can lead to the development of powerful drugs that help to conquer CRC.

Chapter 1

INTRODUCTION

Cancer is known as the disease in which cells evade apoptosis or cellular suicide and acquire proliferating and transforming capacity. Apoptosis consists of a program of cellular self-destruction that involves cascades of gene families, signaling pathways and specialized sub-cellular microenvironments. Regulation of apoptosis is essential for remodeling organisms during embryonic and fetal development, removing unnecessary and archaic histological structures, and imparting resilience to specialized tissue compartments, such as the central nervous system. In the adult organism, apoptosis maintains the homeostasis of differentiated tissues by regulating the balance between cell proliferation and cell death (Altieri 2003).

The major group of proteins that regulates cell death is the inhibitor of apoptosis (IAP) family of proteins. The IAP family of proteins is identified by the Baculovirus IAP repeat (BIR) domain which is 70-amino-acid zinc-finger fold as well as the RING domain, which is a cysteine rich sequence that is capable of binding two zinc molecules. A caspase-recruitment domain also can be seen in some IAP family members. In humans, eight members of the IAP gene family have been identified including XIAP, c-IAP1 and c-IAP2 (Salvesen 2002).

1.1 Background on survivin

Survivin (*BIRC 5*) is one of the smallest (16.5 kDa) members of the family of IAPs. It carries a single BIR domain and an extended carboxy-terminal α-helical coiled-coil, but lacks a RING domain. There are three alternatively spliced forms of the survivin

protein, survivin, survivin B, and survivin ΔEx3. The wild- type survivin gene has a genomic organization consisting of three introns and four exons in the human (Li, Ambrosini et al. 1998). In addition, two survivin isoforms are generated through alternative splicing by the insertion of an additional exon 2 (survivin-2B) or the removal of exon 3 (survivin-ΔEx-3) (Figure1). Survivin variants are thought to modulate cancer development through their differential expression. It is suggested that survivin-2B is a natural antagonist against wild type survivin and survivin ΔEx3 (Li 2005).

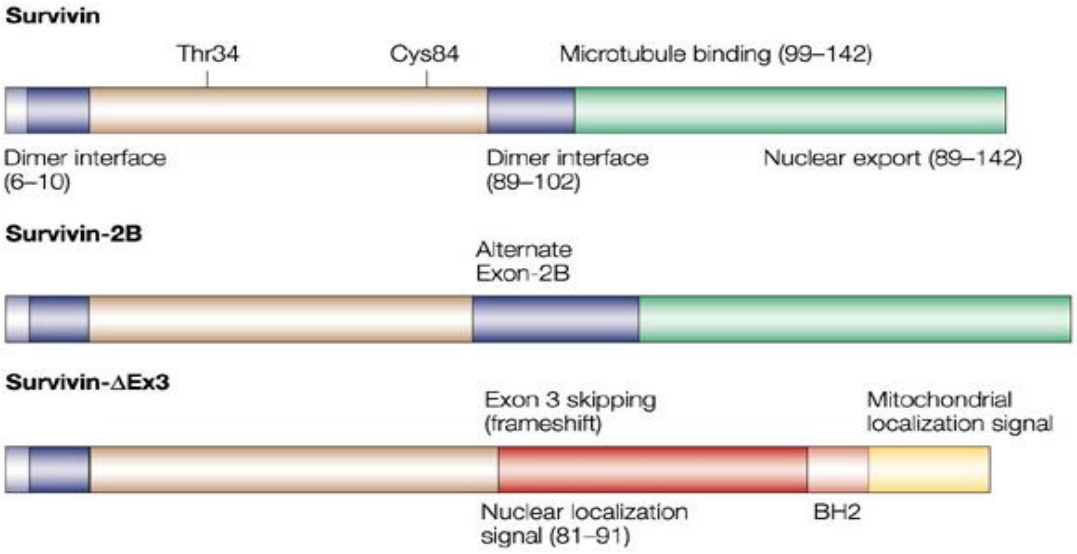

Figure 1: Structure of survivin proteins generated by alternative splicing
Organization of survivin, and its alternatively spliced variants, involving insertion of an alternative exon (survivin-2B), or deletion of exon 3 (survivin-ΔEx3).
Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] (Altieri 2003), © 2002

It has been shown previously that survivin expression is regulated by the adenomatous polyposis coli (*APC*) protein through the *β-catenin*/T-Cell Factor (*TCF*)-*4*

pathway (Zhang, Otevrel et al. 2001). It is known that an *APC* mutation, which activates *β-catenin* and *TCF-4*, is the main pathogenic mechanism leading to familial colorectal cancer (CRC) (Fodde, Smits et al. 2001). This mutation in *APC* leads to the inability of *APC* to bind to and induce the degradation of *β-catenin*. The accumulation of *β-catenin* in turn activates survivin, which inhibits apoptosis. Further, by virtue of its binding to aurora B kinase, (*ABK*) (Wheatley, Carvalho et al. 2001), survivin forms a protein complex that promotes cell division (Figure 2) (Boman et.al.2004).

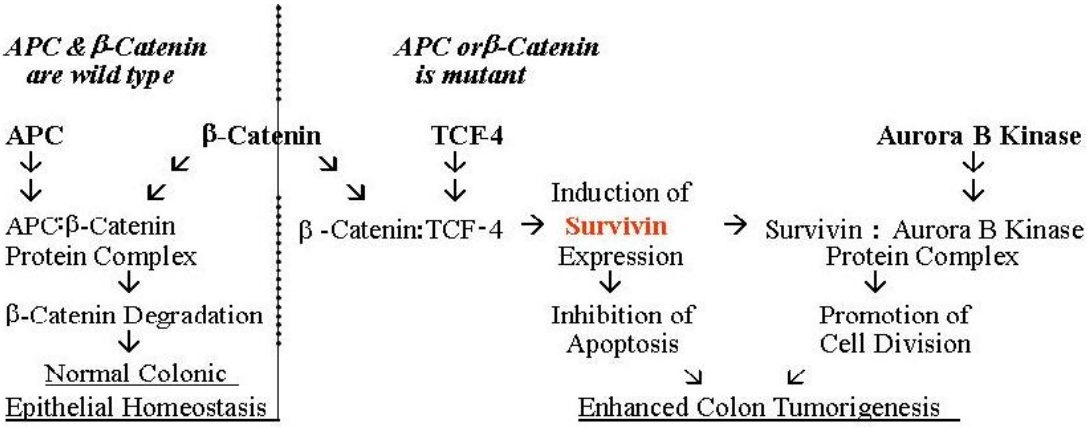

Figure 2: Schematic of the *β-catenin*/*TCF-4*/survivin pathway
Possible mechanism involved in colon tumorigenesis that is caused by *APC* or *β-catenin* mutation that leads to inhibition of apoptosis and promotion of cell proliferation. Permission Requested from AACR Publications [Cancer Research] Letters to the Editor, Boman BM, et al,64:776-779, © 2004.

Survivin, which is over-expressed in many cancers including CRC, has a major regulatory function in cell division and apoptosis. It has been demonstrated previously that inhibition of apoptosis by survivin alone or in conjunction with *BCL-2* is a predictive criterion in CRC (Kawasaki, Altieri et al. 1998). This relationship has been

elaborated further (Sarela, Macadam et al. 2000) using a Kaplan-Meier-type survival analysis for patients with curatively resected CRCs (stages I, II, and III) stratified according to the level of expression of survivin. It was found that survivin over-expression occurs in >70% of CRC cases, this predicts poor patient survival and higher tumor recurrence rates (Figure 3).

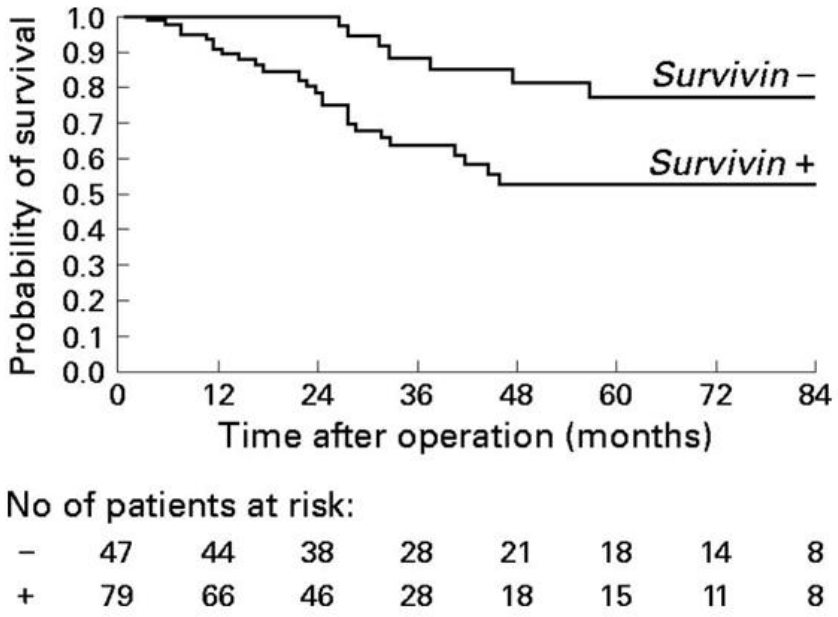

Figure 3: Kaplan-Meier survival plot for survivin
Patients with curatively resected colorectal carcinomas (stages I, II, and III) stratified according to tumor expression of survivin. "Adapted by permission from BMJ Publishing Group Limited. [Gut,Sarela,A.I.,et.al,46,645-650, ©2000]"

It is known that survivin is over expressed in >70% of CRC compared to normal colonic epithelium and is a *TCF-4* target gene. Wild type survivin has been reported to be expressed highly in keratinocyte stem cells (SC), but its mRNA decreased in transit amplifying cells and disappeared in post-mitotic cells (Marconi, Dallaglio et al.

2007). Studies previously done indicate that colonic SC overpopulation promotes colon tumorigenesis, which led to investigation of a possible role for survivin in the SC overpopulation. It was found that while survivin staining is localized to the lower part of the normal crypt, colon-carcinomas show strong staining for survivin throughout the colonic epithelium (Boman, Walters et al. 2004).

Survivin is known to interact with a high number of signaling molecules, regulators, and transcriptional networks that, are involved in its functions directly or indirectly (Altieri 2008). To appreciate the complexity it is not enough to think of survivin alone, which becomes apparent after performing network analyses that link survivin to multiple signaling circuits. A systems-biology approach has to be used to reiterate the extraordinary heterogeneity of tumor cells involving self-acting sub-systems, redundancy, and many layered organization. This model suggests survivin as a nodal protein involved in multiple cellular networks, from which coordinate signaling pathways branch out to regulate different aspects in cellular homeostasis. This led us to predict that there may be networks of genes that are simultaneously regulated along with survivin by a network of transcriptional regulators.

1.2 Identification of genes that are expression-correlated with survivin

To understand the regulatory mechanisms involved in expression of survivin, our lab set out to identify the genes or group of genes that are expression-correlated with survivin. Two of the microarray databases that first became publicly available to the scientific community were analyzed for gene expression patterns that correlated with the expression of survivin. Databases included: **1)** the murine GI tumor database (69 tumor samples) from the Vanderbilt Cancer Center (http://genometrafac**.**cchmc**.**org; Dr RJ Coffey) which involved a cDNA Microarray (20,000 genes) study; and **2)** a hematological malignancy database involving 155 human leukemia samples from the St

Jude's Medical Center and an Affymetrix microarray (26,000 genes). The data were normalized and analyzed using GeneSpring 7.0 (Silicon Genetics). Gene expression patterns were compared with survivin expression across all tumor samples in each database using a standard correlation distance measure (http://www.cincinnatichildrens.org/research/cores/informatics). Analysis of the human hematologic malignancy microarray database revealed that 40 genes are expression-correlated with survivin. Analysis of the murine GI tumor database revealed that 119 genes are expression-correlated with survivin.

In another independent analysis, the Affymetrix CEL files for data sets containing healthy and neoplastic human colon samples were obtained when they became available from the Gene Expression Omnibus (Barrett and Edgar 2006) and Array Express (Brazma, Parkinson et al. 2003). After performing background adjustment, quantile normalization (the method of matching up varying values across all arrays so that the smallest value on each array is identical, the second smallest is identical, and so forth. The smallest value for one array might represent a gene other than the smallest value on another array), and summarization by computing the robust multichip average (RMA) (Brazma, Ikeo et al. 2003) for the entire sample set using only probe-sets in common between the HGU133A and HGU133plus2 arrays, the combined colon data sets were used to identify genes with medium to strong gene expression that correlated with survivin expression (with positive correlation above 0.65 and negative correlation below 0.65). Correlated gene sets were identified separately in the subsets of healthy colon, colon tumors, and the combined set of normal, adenoma, and cancer samples. Combined analysis of the human colon database revealed that 62 genes are expression-correlated with survivin (Figure 4A).

Initial inspection of genes that are expression-correlated with survivin showed that the vast majority (>90%) are relevant to cell-cycle regulation and cancer. Indeed, it is known that survivin is expressed preferentially during mitosis (Mita, Mita et al. 2008) and colon cancers have survivin over-expression (Fukuda and Pelus 2006) and display increased mitotic figures (Altieri 2003). Categories of genes included centromeric proteins, kinesin-like proteins, DNA synthesis licensing proteins, cyclins, and cyclin-dependent kinases. Two of the survivin expression-correlated genes encode proteins (INCENP and ABK) that bind to survivin and function in survivin's downstream pathway by forming a protein complex that activates ABK and catalyzes mitosis.

As the expression-correlated analysis showed that there is a specific cohort of genes that are expression-correlated with survivin, it was proposed that the expression of this set of genes is co-regulated by a common set of transcription factors (TFs). To determine if these genes are transcriptionally co-regulated, the promoter regions of the genes were analyzed to find significantly enriched and shared transcriptional regulatory elements (TREs), which provided information on candidate TFs that are involved in this co-regulation.

1.3 Transcriptional Regulatory Network Analysis (TRNA) and Promoter Analysis and Interaction Network Toolset (PAINT)

Transcriptional regulatory network analysis (TRNA) of a set of functionally related genes involves finding the TF binding sites that are present in gene promoters and checking to determine if there are any binding sites in common across the genes. Commonality of TF binding sites in promoters of functionally related genes suggests that the TFs binding to those sites play a role in regulating the expression of the related genes, thus forming a testable gene regulatory hypothesis. The software Promoter Analysis and Interaction Network Toolset (PAINT) (Gonye, Chakravarthula et al. 2007) was used to

automate TRNA. PAINT bridges the gap between transcriptional regulation and microarray data, given that genes showing similar patterns of expression can be tested for significant enrichment of TF binding sites in their promoters. For any functionally-related group of genes, PAINT retrieves the cognate genomic promoter sequences, analyzes them for the presence of known TF binding sites, and compares these results to those obtained from random gene groups to test analytically for statistically significant enrichment of binding sites. Furthermore, given that TFs often function in cooperative regulatory modules, it was also tested for statistically significant co-occurrence of binding sites in gene promoters. The tool can also produce output in various formats suitable for use in external visualization and analysis software. PAINT can be accessed at www.dbi.tju.edu/dbi/tools/paint/.

1.4 Identification of enriched TREs in promoter regions of survivin expression-correlated genes

The resulting gene subsets were used for further analysis using PAINT software (Figure 4A) (Dr. Gregory E. Gonye and Dr.Adam Ertel, unpublished data). PAINT results clearly identified several TF binding sites over- and under-represented on the survivin-correlated gene set (Table 1) (Figure 4B). When cluster membership was taken into consideration, some of these sites appear to be restricted to specific expression clusters (Figure 4C). Hierarchical clustering was also done across the samples based only on the survivin-correlated and anti-correlated genes, further supporting the likelihood of co-regulated groups of genes in this set.

The clustering analysis showed that many of the clusters included TREs associated with cell-cycle genes including genes associated with mitosis such as centromere proteins and proteins involved in spindle body formation. For example it has been shown that the promoter of CDK1 contains TREs called cycle dependant elements

(CDE) and cycle homology regions (CHR) (Badie, Itzhaki et al. 2000). From this TRE analysis it can be proposed that the cognate TFs may play a major role in the regulation of the expression of survivin and its expression-correlated genes. Hence a panel of 11 cognate TFs was selected from the significantly enriched TREs.

These candidate TFs included *E2F* family of cell-cycle genes *E2F4* and *E2F1*, tumor suppressor gene *TP53*, tissue developmental genes *PAX6*, *MYB*, *NKX2-5*

Table 1:Over-represented TREs in promoters of survivin expression-correlated genes

Normal colonic epithelium	**Colorectal cancer**
CREB	PPARα:RXRα
CRE:c-jun	NFY
C- jun	PAX6
GFI	ETF
E2	C-ETS1: p54
CCAAT box	ZF5
E2F	V- *MYB*
	NKX2-5

and *NFE2*. Master TF genes that control other TFs like nuclear transcription factor Y subunit alpha (*NFY-A*) and *ELK1* were included as was upstream activating factor (*USF1*) and *TDP2*.

From this panel of specific TFs a hypothesis was generated: *The specific TFs corresponding to significantly overrepresented TREs in promoter regions of survivin and its expression-correlated genes are expressed differentially in normal versus malignant colon tissues.*

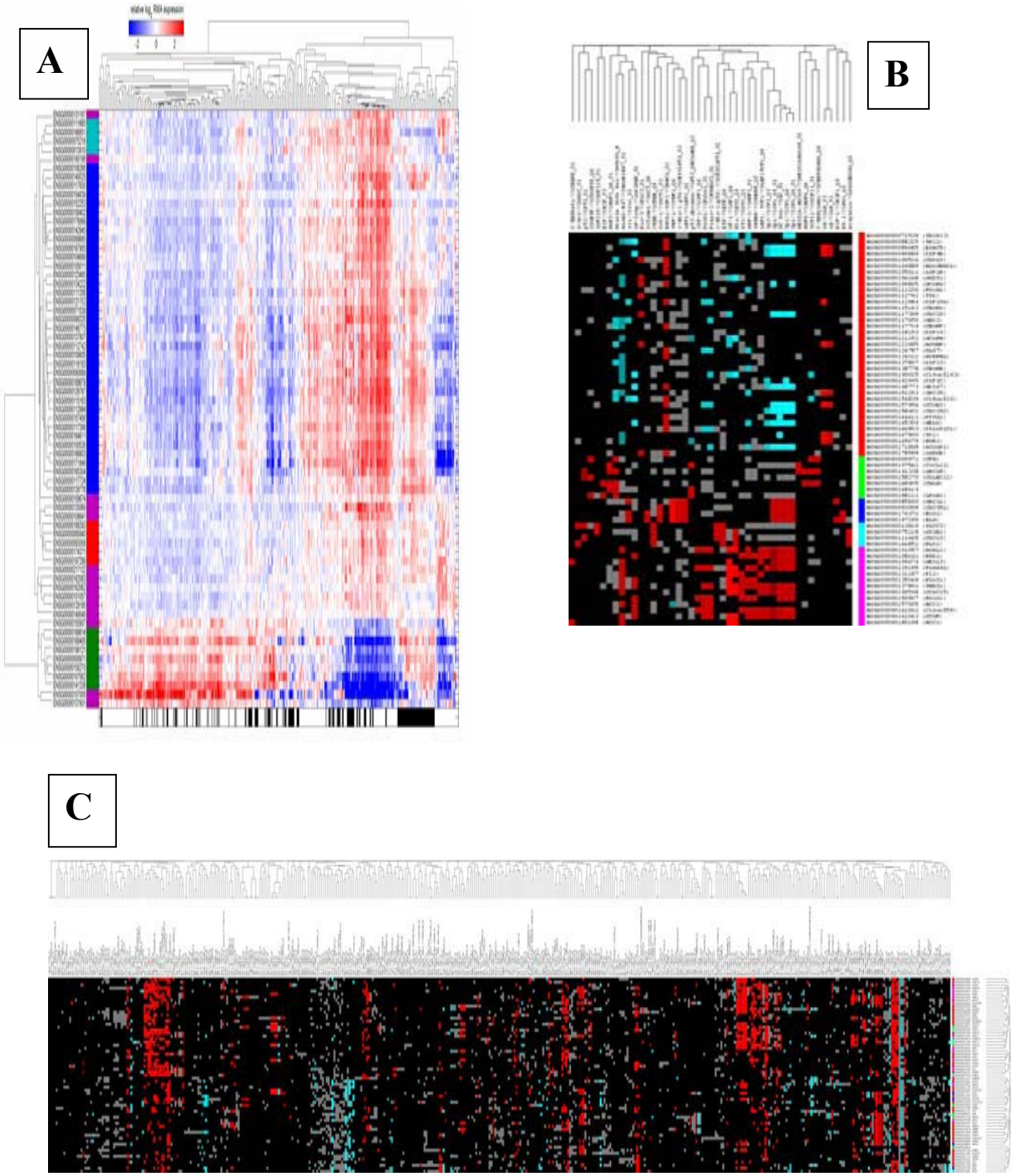
A
B
C

Figure 4:Large-scale network analysis of survivin correlated genes

Survivin-correlated or anti-correlated genes based on normalized expression across normal, adenoma and cancer samples were used to group genes into clusters (Panel A). Red represents increased and blue decreased levels of expression relative to the mean level of expression. On the vertical "gene" axis, genes are organized into five groups based on their cluster patterns, with blue, green, red and cyan representing groups defined by clusters, ordered largest to smallest, and magenta representing outliers On the horizontal axis white represents normal colon, grey represents adenoma and black represents cancer. The gene list and cluster membership was used to generate predicted gene networks with PAINT (Panel B). Panel C shows a subnetwork of only those most enriched TREs organized by cluster membership. Clusters were determined from data in panel A (Dr. Gregory E. Gonye and Dr.Adam Ertel, unpublished data). This figure is shown to illustrate patterns that can be seen when a large set of genes is surveyed; it is not meant to show details, which is why the axes are not legible at this scale.

Chapter 2

DETERMINING THE PRESENCE OF CANDIDATE TRANSCRIPTION FACTORS AT THE MRNA LEVEL IN NORMAL AND MALIGNANT COLON TISSUE

2.1 Introduction

Large-scale network analysis of survivin correlated genes done in our lab has shown that there is a set of genes that are expression-correlated along with the survivin gene (*BIRC 5*). It was predicted that the expression of this set of genes is co-regulated via a set of TFs. To elucidate if these genes are co-regulated, promoter regions of the genes were analyzed for common TREs which provides information on TFs that might be involved in the co-regulation.

It has been reported previously that several TFs are involved in the regulation of survivin expression. In non-transformed embryonic fibroblasts, *E2F* family of TFs (*E2F1, E2F2, E2F3*) have been shown to bind to survivin's promoter and induce transcription of the survivin gene (Jiang, Saavedra et al. 2004). *E2F* family of TFs shares a DNA binding domain and bind to overlapping sets of target promoters. Also, *E2F* proteins associate with another TF called DP2 forming heterodimeric complexes that bind to DNA (Girling, Partridge et al. 1993). In *Caenorhabditis elegans* and *Drosophila melanogaster*, protein complexes have been isolated that contained a retinoblastoma protein, DP protein, an *E2F* subunit, *MYB* and a group of *MYB* interacting proteins (Lewis, Beall et al. 2004). There has also been a report of a downstream repression site (DRS) that is closely linked to an *E2F* site, which is needed for B-*MYB* transcription (Catchpole, Tavner et al. 2002). In gastric cancers, survivin has been shown to be

overexpressed along with the anti-apoptotic protein BCLl-2 and a mutant form of *TP53* (Lu, Altieri et al. 1998). Inflammatory bowel disease, (including chronic ulcerative colitis, which can lead to CRCs), is associated with over-expression of survivin along with the proto-oncogene c-*MYB* (Svec, Musilkova et al. 2009). In non-small cell lung cancer, *E2F1* has been shown to be correlated positively with survivin expression (Huang, Liu et al. 2007). All these reports suggest that specific TFs are involved in regulation of numerous proteins including regulation of other TFs as well. I hypothesized that survivin along with its expression-correlated genes is regulated by a specific set of TFs.

Large-scale network analysis suggests the possibility that survivin and its expression-correlated genes are regulated by the same TFs. In the bioinformatics analysis using PAINT (Vadigepalli, Chakravarthula et al. 2003) a set of TREs corresponding to specific TFs was identified. These TFs were considered further as candidate TFs that regulate survivin and its set of expression-correlated genes.

Many of the candidate TFs are relevant to cell-cycle regulation and mitosis. The candidate TF panel includes *E2F1* and *E2F4* which are *E2F* family members and regulatory proteins of the S-phase entry of cell-cycle (Dimova and Dyson 2005). *MYB* is a proto-oncogene that plays a role in oncogenesis (Ramsay and Gonda 2008). Wild type *TP53* is a tumor suppressor but is mutated in many types of cancers. In a study with 10 colorectal carcinoma cell lines, six of the cell lines showed high expression of mutant *TP53* (Rodrigues, Rowan et al. 1990). The homeobox gene *NKX2-5* is a known marker for cardiogenesis in vertebrate embryos and is expressed in cardiac progenitor cells before differentiation and through adulthood in mice (Lints, Parsons et al. 1993) and in chicken (Schultheiss, Xydas et al. 1995). Also TFs such as *NKX2-5* and *ELK1* are

involved in tissue development (Schwartz and Olson 1999) and differentiation (Seth and Watson 2005).

Little is known about how these specific TFs regulate expression of survivin in colon tissues. As discussed above, we found that the TRE elements corresponding to these candidate TFs were over- or under-represented in the survivin correlated gene data set. This finding led to my immediate hypothesis: *Specific TFs in the set of candidate TFs are differentially expressed at the mRNA level in normal versus cancerous colonic mucos*a

The next logical step is to verify whether the TF gene expression changes correlate with changes in mRNA as well as protein levels in normal versus cancer tissues. Even though cell lines might be used to evaluate this possibility, actual analysis of tissue will reflect more of the *in vivo* situation.

2.2 Materials and Methods

2.2.1 Patient samples

Five matched normal and CRC tissue samples were collected from Christiana Hospital after patient consent under an **I*RB*** approval. Patients included both males and

Table 2: Patient samples

Patient Number	Sex	Age	Part
0918	Male	40	Sigmoid
0924	Male	59	Recto-Sigmoid
0923	Female	78	Right Colon
0938	Male	49	Recto-Sigmoid
0921	Female	56	Obstructing Sigmoid cancer

females between ages 40 and 80 (Table 2). The samples were immersed in RNA Later (Ambion) and kept at -80°C until further processing.

2.2.2 Isolation of total RNA from normal and malignant colonic tissue

Total RNA was isolated from the malignant colonic tissue as well as surrounding normal tissue by homogenizing the tissue sample and later precipitating the total proteins using the Trizol Reagent (Invitrogen) and chloroform. Following phase separation, total RNA that remained exclusively in the aqueous phase was transferred to a fresh centrifuge tube and precipitated using Isopropyl alcohol. After centrifugation, the precipitated RNA was washed using 75% ethanol and later dissolved in 100µL of Molecular Grade water. The integrity as well as the concentration of the RNA samples was verified using a Bioanalyzer 2100 (Agilent Technologies) and Nanodrop ND100 (Thermo Scientific).

2.2.3 Quantitative Real time Polymerase Chain Reaction (Q-RT-PCR)

The total RNA (20ng/µL) was reverse transcribed using an oligodT primer (0.5µg/µL), dNTPS and Superscript II Reverse Transcriptase (Invitrogen) by incubating at 42°C for 50 minutes. The contents of the tube were diluted to make approximately 1ng of cDNA/µL.

Using quantitative real-time polymerase chain reaction (Q-RT-PCR), the cDNA was analyzed for the increase in the fluorescence signal intensity of the reporter dye Sybr Green with Rox as the passive reference (iTaq SYBR Green Supermix, BioRad Laboratories) using TF gene-specific primers (Table 3). The TF gene specific primers were designed with intron spanning primers using NCBI/primer blast. The formation of the PCR product was detected using an ABI Prism 7000 series Q-RT-PCR machine. The data was analyzed using ABI 7000 system software. The level of mRNA of TFs relative to Ribosomal protein L-19 (*RPL-19*) was calculated using the formula Fold change = 2^{-}

$^{\Delta\Delta Ct}$ where $\Delta\Delta Ct = (Ct_{\text{Transcription factor}} - Ct_{RPL\text{-}19})$ cancer tissues – $(Ct_{\text{transcription factor}} - Ct_{RPL\text{-}19})$ adjacent normal tissue, in which Ct is the number of cycles where the fluorescence exceeds a set threshold. Reactions without target cDNA were used as negative controls.

Table 3: Sequences of gene specific primers used in quantitative PCR reactions

Candidate Gene	Forward Primer	Reverse Primer
E2F4	GGGTGGTCCAGCCCTGAT	AAGCCGAAATGAAGAGAGGGTTAT
PAX6	AACCTGGCTAGCGAAAAGCA	CCCGTTCAACATCCTTAGTTTATCA
NKX2-5	GACCCTGAGTCCCCTGGATT	GCGCGTGGGACAGAAAAA
MYB	CTCAGACACCCTCTCATCTAGTAGAAGA	CTCAGCAACAATTCCAGATTCATC
ELK-1	GCCAAGCTCTCCTTCCAGTTT	ATCCACGCTGATAGAAGGGATGT
NFE2	TCTCCATATCCCATTCCCTGTAGA	TCTTTGGACATCATTTCGTTGAA
USF-1	TGAACTGAGGCCCTGTGATATG	CAGTGCACGTCCACATTGTG
NFY-A	AATAAACTGCAGACTGAAAGAAACATG	ACAACCCGTTCTGGGAACAG
TDP2	GCCACCTTACCCCAGTCAAG	CCCACTTGCTAAGGCCACTTC
E2F1	CCCAACTCCCTCTACCCTTG	GAAAGTGAGGGAGGGAGACAGA
TP53	TGTCCTTCCTGGAGCGATCT	CAAACCCCTGGTTTAGCACTTC

2.2.4 Validation of *RPL-19* as the reference gene

The Q-RT-PCR analysis was started first using the common reference gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). But soon it was noticed that *GAPDH* had different mRNA gene expression between CRC and normal epithelial tissues. There have been many papers published regarding the assumption in the use of

housekeeping genes that expression of the genes remains constant in the cells or tissues under investigation (de Kok, Roelofs et al. 2005). Even though *GAPDH* is considered as a suitable reference gene for many systems, in many cases it shows a difference in expression between tissues (Barber, Harmer et al. 2005), specifically between normal and tumor tissues.

My analysis using *GAPDH* as reference gene gave a significant difference in expression between normal and tumor tissue (Figure.5). Hence *GAPDH* had to be discarded and another reference gene had to be determined. Further analysis proved that Ribosomal protein L-19 (*RPL-19*) was suitable as a good reference gene in a CRC context (Figure 6). *RPL-19* was observed to give constant expression of genes at the mRNA level in tumor tissue as well as adjacent normal colonic epithelium. Hence *RPL-19* was selected as the reference gene for the analysis of fold change for the mRNA level gene expression. I also examined other common house-keeping genes like TBP, HPRT, TPT, β-actin, and UBC (Table 4).

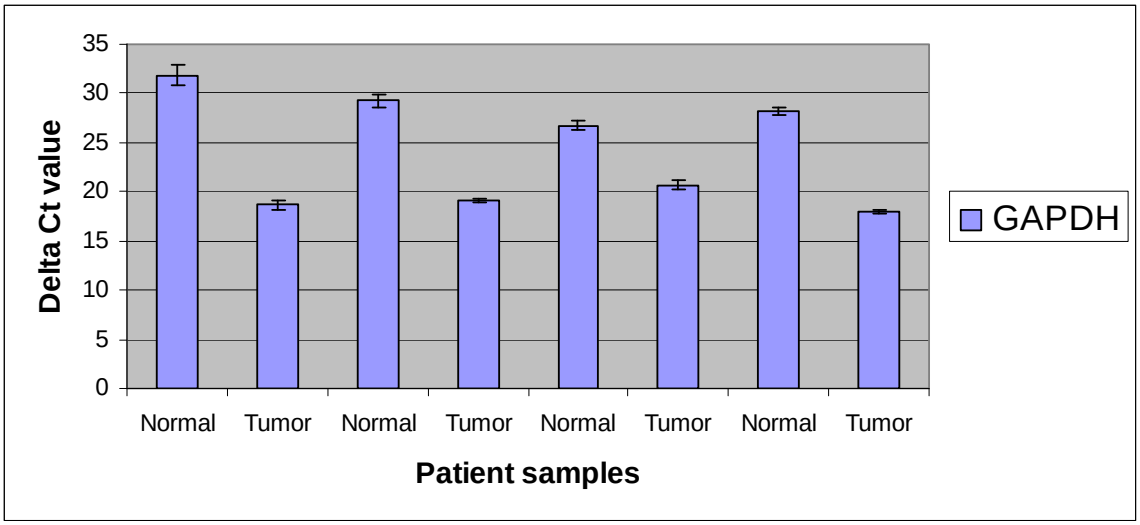

Figure 5: Fold change for *GAPDH* between normal and tumor samples

Raw Ct values for *GAPDH* between four tumor and matched adjacent normal tissue for four patients. Error bars represents standard deviation between triplicates

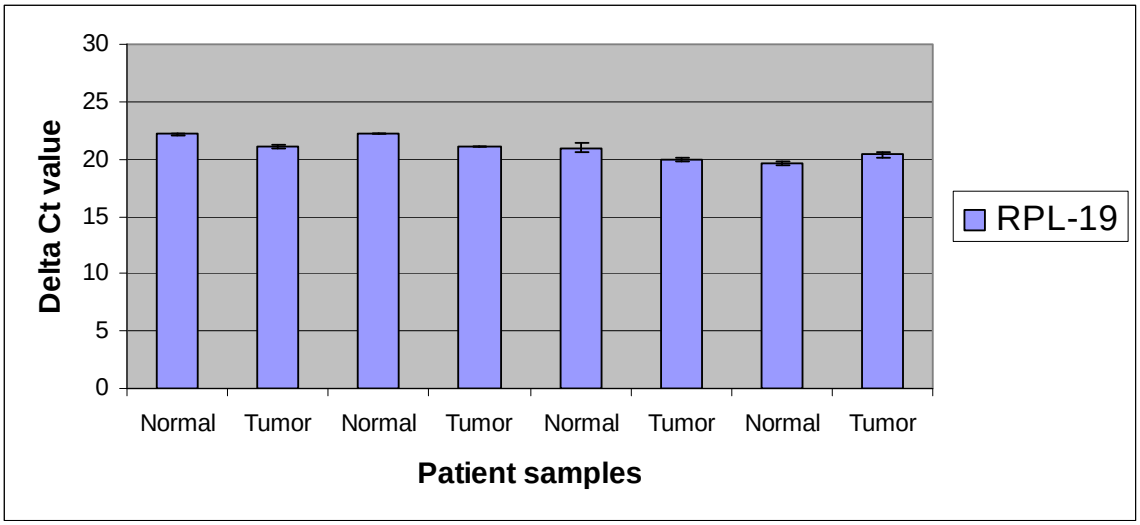

Figure 6: Fold change for *RPL-19* between normal and tumor samples

Raw Ct value for *RPL-19* between four tumor and matched adjacent normal tissue for four patients. Error bars represents standard deviation between triplicates.

Table 4: Sequences of reference primers used in quantitative PCR reactions

Candidate Gene	Forward Primer	Reverse Primer
RPL-19	GAGGAGAATGAGGATTTTGCG	GGCTGTGATACATGTGGCG
GAPDH	GCTGAGTATGTCGTGGAGTC	TTGGTGGTGCAGGATGCATT
β-Actin	CGTACCACTGGCATCGTG	GTTTCGTGGATGCCACAG
TBP	TATAATCCCAAGCGGTTTGC	GAAAATCAGTGCCGTGGTTC
UBC	GATTTGGGTCGCGGTTCTT	TGCCTTGACATTCTCGATGGT
HPRT	AAGCTTGCTGGTGAAAAGGA	TCAAATCCAACAAAGTCTGGC
TPT1	GATCGCGGACGGGTTGT	TTCAGCGGAGGCATTTCC

2.2.5 Statistical analysis

Paired t-test was done between ΔCt value for normal and tumor tissue for each of the TFs for the five samples. A p-value less than 0.05 was considered significant.

2.3 Results

The set of TFs that was selected for further analysis can be divided into two groups, one consisting of cell-cycle-associated factors and the other related to tissue development and differentiation. Table 5 elaborates the physiological function of each selected TF.

Table 5: Candidate transcription factors and their physiological function

Transcription factor	Function
E2F4	Controls cell-cycle and tumor suppressor proteins
E2F1	Controls cell-cycle and tumor suppressor proteins
ELK1	Nuclear target for Map-kinase, ETS family member
MYB	Oncogene, haematopoesis
USF1	Familial combined Hyperlipidimia
PAX6	Development of Eye and Nervous system
NKX2-5	Cardiogenesis and cardiac differentiation
TP53	Controls cell-cycle, tumor suppressor protein
NFE2	Hemoglobin production
NFY-A	Highly conserved transcription factor, regulates other TFs
TDP2	Stimulates transcription of *E2F*s

2.3.1 mRNA expression levels in malignant colonic tissue and adjacent normal mucosa

To analyze the mRNA expression pattern of the candidate TFs in malignant CRC tissue and surrounding normal tissue, matched normal and tumor colorectal tissue samples from five patients including both males and females aged from 40-80 years old were selected. Q-RT-PCR (40 cycles) was done for the specific TF using gene -specific primer pairs. The Ct value from the Q-PCR machine was used for further analysis. Negative controls without gene specific primers did not give any PCR product. The degree of gene expression was quantitated by calculating ΔCt values. ΔCt is the difference in the cycle threshold values between candidate TFs and the internal reference gene *RPL-19*. ΔCt was calculated for both normal and tumor tissues. A high ΔCt value correlates to low expression of the gene while a low ΔCt value is associated with high expression of the gene.

For each matched normal and tumor tissue sample ΔCt values were obtained by performing triplicate Q- PCR reactions. We observed that TFs, *E2F4*, *MYB*, *NFE2*, *USF1*, *NFY-A*, *TDP2* and *TP53* (Figure 7 and Figure 8) showed a higher ΔCt value for normal and a lower ΔCt for tumor samples. This indicates that these six TFs are expressed at a higher level in tumor tissues compared to normal tissues at the mRNA level. *PAX6* and *NKX2-5* showed an almost equal ΔCt value, indicating that the level of expression at the mRNA level in malignant colonic tissue was not changed compared to normal epithelium. *E2F1* gave a high ΔCt value in both normal and tumor tissues, suggesting that it was expressed at a basal level in both normal and cancer tissues.

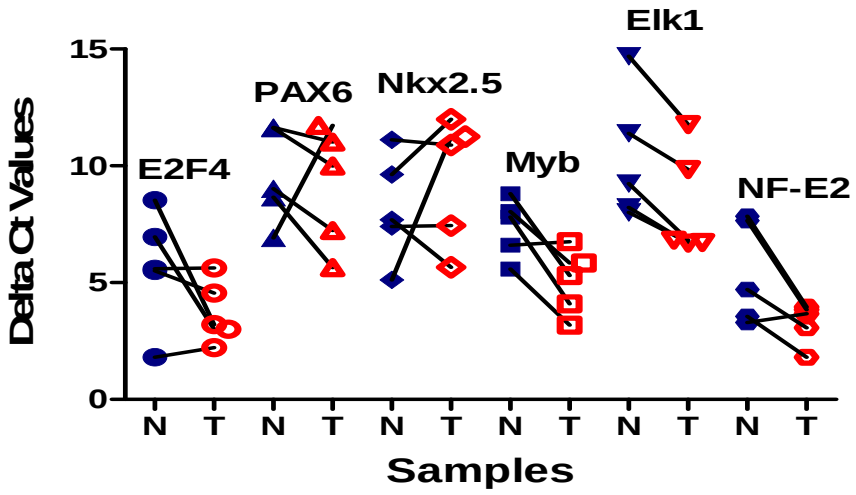

Figure 7: ΔCt values for the TFs *E2F4*, *PAX6*, *NKX2-5*, *MYB*, *ELK1* and *NFE2*. ΔCt is the difference in the cycle threshold values between specific TFs and the normalizing gene *RPL-19*. *MYB* and *ELK1* gave a significant p-value of less than 0.05 based on paired t-test between normal and tumor ΔCt.

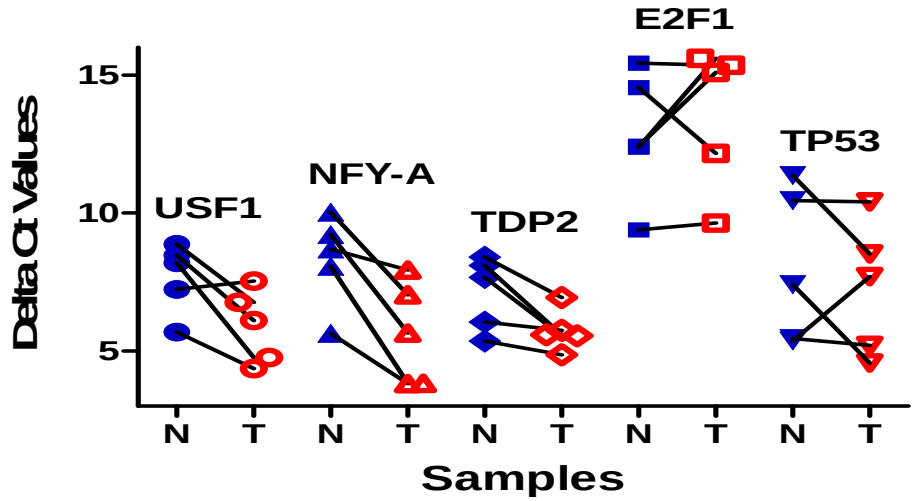

Figure 8: ΔCt values for the TFs *USF1*, *NFY-A*, *TDP2*, *E2F1* and *TP53*. ΔCt is the difference in the cycle threshold values between specific TFs and the normalizing gene *RPL-19*. *NFY-A* and *TDP2* gave a significant p-value of less than 0.05 based on paired t-test between normal and tumor ΔCt.

After the calculation of ΔCt values, further analysis was done by calculating the mean fold change for each TF mRNA between normal and tumor tissues (Figure 9). The mean fold change for each TF was calculated by averaging the fold change for the five patients. It was seen that *NFY-A* and *E2F4* gave the highest fold change of 7-11X. The TFs, *MYB* and *NFE2* gave a fold change of around 6X, while *USF1* gave a gene expression change ~ 4X. *E2F4*, *ELK1*, *TDP-2*, and *PAX* 6 showed fold changes between 2-3X. The lowest fold change was observed for *NKX2-5* as well as *E2F1* each of which was <1X.

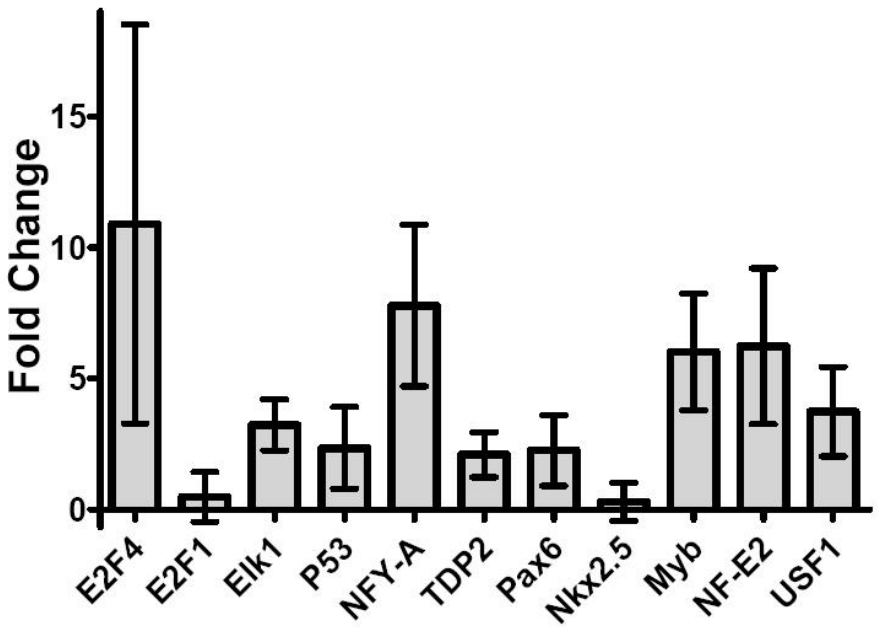

Figure 9: Mean fold change for candidate TFs across five patients between normal and tumor colonic mucosa. Fold change was calculated using the Δ-Δ Ct method. Error bars were calculated by measuring the standard error of the means.

2.3.2 Statistics

It was observed that TFs *MYB*, *NFY-A*, *ELK1* and *TDP2* gave a significant p-value of <0.05 when a paired t-test was performed between ΔCts of normal and tumor samples.

2.3.3. Analysis of wild type survivin at the mRNA level

To verify whether the increase in expression of candidate TFs correlates with the expression of wild-type survivin at the mRNA level, Q-RT-PCR analysis was performed for wild-type survivin for the same patients (Figure 10). It was observed that there was an increase in the expression of survivin at the mRNA level for the same patients which showed positive correlation with the increase in expression of candidate TFs at the mRNA level.

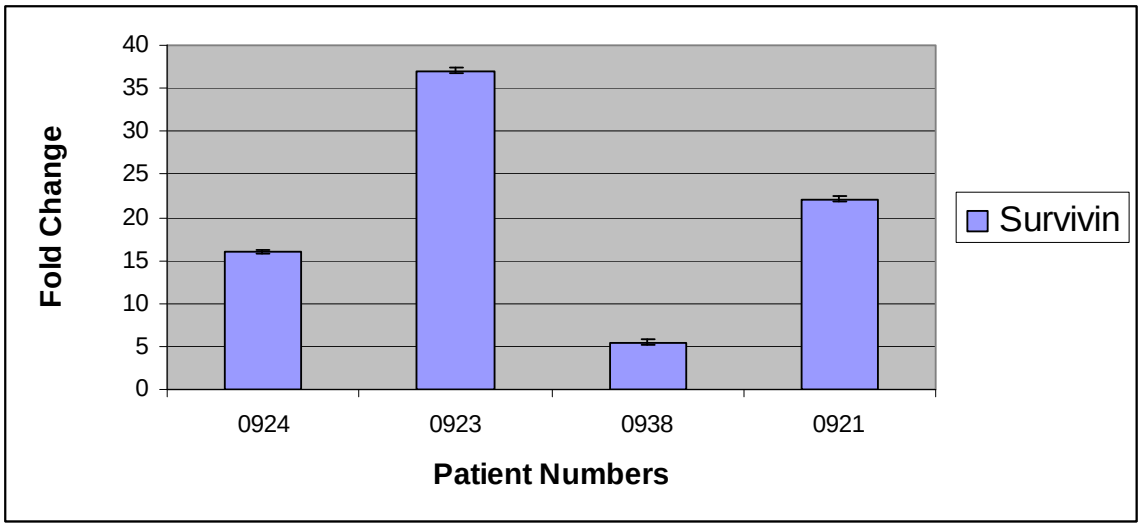

Figure 10: Mean fold change for survivin across four patients between CRC tissue and normal colonic mucosa. Fold change was calculated using the Δ-Δ Ct method. Error bars were calculated by measuring the standard deviation for the technical triplicates

2.4 Discussion

There have been reports using specific gene signatures to predict aggressive cancer behavior (Hernandez-Vargas, Lambert et al.). Gene signature also helps in revealing the biological processes involved in cancer occurrence and progression (Hirsch, Iliopoulos et al.). Microarray analysis of urinary bladder cancers of mice expressing transgenic survivin showed global changes in gene expression with up-regulation of extracellular matrix and inflammatory genes compared to normal mouse urinary bladder (Salz, Eisenberg et al. 2005).

Survivin has been shown to be a predictive tumor marker for patient survival and tumor recurrence in CRC (Sarela, Macadam et al. 2000), but little is known about the TFs that play a role in the regulation of survivin as well as several of the other genes that are expression-correlated with survivin. This led me to hypothesize that the expression of this set of genes may be co-regulated by a common set of TFs. To identify enriched regulatory elements supporting my hypothesis of co-regulation, the promoter regions of the expression-correlated genes with survivin were analyzed to identify shared TREs. This provided information on cognate TFs that can bind to the TREs. Eleven candidate TFs were selected that corresponded to those TREs showing statistically over-representation in the set of expression-correlated genes. These TFs were later evaluated for mRNA as well as protein-level expression between normal and CRC samples. As a logical first step, the expression of mRNA was investigated for differential expression between normal and tumor samples.

Our results showed that, the entire set of candidate TFs except for *E2F1* and *NKX2-5*, showed an increased level of mRNA expression in cancer tissue compared to normal epithelium. The increase in expression of *TP53* is consistent with the previous reports. It is known that *TP53*, which is a tumor suppressor gene, becomes mutated in

many cancers, which leads to increased levels and contributes to proliferation and transformation to cancer (Hussain and Harris 1999). The absence of wild- type p53 in mice denotes susceptibility to various tumors (Donehower, Harvey et al. 1992). P53 acts to directly and indirectly repress transcription, which is critical to its role as a tumor suppressor (Ho and Benchimol 2003). This can become a factor that contributes to tumorigenesis because as the pathways become dysregulated many transcriptional changes take place. Deregulation of transcription may result in the failure to express genes responsible for cellular differentiation, or in the transcription of genes involved in proliferation, through the incorrect expression or induction of positively acting transcription factors and nuclear oncogenes. In our TF mRNA analysis, *TP53* expression was increased >2 fold. This leads us to hypothesize that *TP53* could have been mutated or dysregulated which leads to the disruption of its tumor suppressor function. This creates accumulation of p53, which is unable to perform its function and leads to increased cell-cycle and cell proliferation. As the tumor suppression of p53 gets negated, proto-oncogenes like *MYB* (Tanikawa, Ichikawa-Iwata et al. 2000) and Myc (Ceballos, Munoz-Alonso et al. 2005) become activated.

The TF *MYB*, which is considered to be an oncogene, was increased six fold in expression in CRC. This correlates with the fact that, in most CRCs, *MYB* is overexpressed at the protein level (Ramsay, Thompson et al. 1992). Over-expression of wild type *MYB* can sequester differentiation and lead to a proliferative cell pathology in mice (Ferrao, Macmillan et al. 1995). Another TF, *NFE2* which is associated with hematopoiesis and platelet differentiation, also showed a fold change similar to that of *MYB*.

NFY-A is another TF that regulates other TFs. Many cancers show an increase in catabolic glucose metabolism. For example, in hepatoma cells, it has been

shown that the mitochondrial Type II Hexokinase, which is a key enzyme in glucose catabolism, has promoter elements where *NFY-A* can bind (Lee and Pedersen 2003). TFs *NFY* and *E2F* are thought to play a role in breast cancer metastasis (Thomassen, Tan et al. 2008). It has been reported previously that *NFY* as well as the *E2F* family of TFs interact with each other via their promoter binding site. *E2F* interacts with *ELK1* to a lesser degree (Tabach, Milyavsky et al. 2005). *NFY-A* expression was eight fold increased in CRC, while *E2F4* and *ELK1* was increased by three fold.

The *E2F* family of TFs plays an important role in S-phase entry of the cell-cycle as well as in numerous other cell proliferative scenarios. Our transcriptional panel included two of the *E2F* family members, *E2F4* and *E2F1*. *E2F4* is considered a transcriptional repressor while *E2F1* is a transcriptional activator (Dimova and Dyson 2005). *E2F1*$^{-/-}$ mice have been shown to be tumor prone (Zhu, Field et al. 2001). In one study it was found that, upon DNA damage, *E2F4* was recruited to the promoter of the dihydrofolate reductase gene (DHFR) that is cell-cycle regulated, while *E2F1* was recruited to the promoter of the apoptosis regulator p73 (Pediconi, Ianari et al. 2003). The DHFR gene is involved in the de-novo synthesis of purines, thymidylic acid, and certain amino acids and plays a critical role in cell growth and proliferation (genecards.com). In our analysis *E2F1* did not show any change while *E2F4* expression was increased three-fold between tumor and normal. This can be due to the fact that *E2F1* is suppressed because an up regulation of *E2F1* can switch on the apoptosis pathway, which would hinder cancer cell proliferation. In contrast, *E2F4* is over-expressed such that there may be an increase in cell proliferation.

Basal transcription of *APC* maintains colon homeostasis and inactivation of *APC* leads to the development of CRC. The promoter of *APC* has nucleotide sequences for E-boxes A, B and M that are functional in cancer cell lines and are seen to bind *USF1*

and upstream activating factor 2 (*USF2*) (Jaiswal and Narayan 2001).As there are three E-boxes involved in the promoter of *APC*, it suggests a high degree of complex transcriptional regulation. The role of *APC* in regulating the c-myc gene expression through a b-catenin/*TCF-4* complex has been examined (He, Sparks et al. 1998). *USF1* and *USF2* are known to antagonize the effects of c-Myc on cellular proliferation (Luo and Sawadogo 1996). The same mechanism can be hypothesized to happen in the case of survivin expression. Under normal conditions *USF1* and *USF2* may bind to the E-boxes of the *APC* promoter, which then regulate the expression of β-catenin. But in CRC, *APC* frequently gets mutated so that *USF1* and *USF2* may not be able to bind to the promoter of *APC*, which disrupts β-catenin regulation that induces survivin and its downstream targets. In the mRNA analysis, *USF1* gave a fold change of less than four. It can also be proposed that there may be some mechanism that induces the over-expression of *USF1* in the cancer compared to the normal tissues.

The paired box (*PAX*) family of TFs encodes nine family members including *PAX6*. *PAX* genes have been reported to be expressed highly in diseases arising in tissues that are highly differentiated (Murer, Caridi et al. 2002). Since colon is a highly differentiated tissue this points to the fact that *PAX* genes might be over expressed in CRC, which is a disease of the colon. In one report, hypermethylation of exon 5 of the *PAX6* gene has been shown to lead to over expression of *PAX6* in human colon and bladder cancers (Salem, Markl et al. 2000). Our analysis of normal and tumor tissues for *PAX6* mRNA gave a fold change of over two.

NKX2-5, which is a master TF involved in cardiogenesis interacts with a major regulator of survivin pathway, β-catenin. In cardiogenesis, *NKX2-5* has been shown to interact with β-catenin and Wnt (Riazi, Takeuchi et al. 2009). Therefore it can be hypothesized that in CRC there is an interaction between *NKX2-5*, Wnt and β-Catenin.

Accordingly, our mRNA analysis of *NKX2-5* did show the presence of *NKX2-5* mRNA, but at a minimal level.

In the present analysis many of the candidate TFs were found to be highly expressed at the mRNA level in CRC compared with normal colonic epithelium. TFs *NFY-A*, *MYB*, *ELK1,* and *TDP2* showed significant changes (p-value of <0.05) based on paired t-test between normal and tumor ΔCt.

Taking all the above mentioned data into context, in normal colon, the master transcriptional regulators like *TP53*, *NFY-A* and *TDP2* may be involved in the regulation of other downstream TFs like the *E2F* family and *USF1*. Also, there are repressor proteins like Retinoblastoma (*Rb*), which keep expression of *E2F* family members in check. In a similar way, p53, which has tumor-suppressor properties, keeps proto-oncogenes like *MYB* repressed. This intricate balance between signaling molecules, TFs, and other proteins maintains normal tissue homeostasis.

But in malignant tissue, this balance is lost due to increased expression of several TFs, particularly ones that regulate proliferation and prevent apoptosis. In many cancers *TP53* gets mutated, which relieves the repression it exerts on TFs like *MYB* that are considered proto-oncogenes. *TP53* mutation or degradation also blocks activation of its downstream protein $p21^{CIP}$ that is an important protein involved in cell-cycle damage checkpoint. Its blockage helps the cell to pass through unregulated cell-cycles which leads to proliferation and tumor formation. This leads to over-expression of these genes which act on other TFs and proteins that induce cellular proliferation like the *E2F* family which inturn induces the expression of several TF cascades (Muller, Bracken et al. 2001). It has been demonstrated that the *E2F* family has many target genes that are involved in cell-cycle control, DNA synthesis, mitosis, DNA repair and apoptosis (Tsantoulis and Gorgoulis 2005).

Our mRNA analysis showed an upregulation of p53 which can be mutated or unable to be sequestered by MDM2. This can lead to inhibition of the tumor suppressor property of p53, which can be a factor in cell proliferation. *E2F4* as well as the proto-oncogene *MYB* also up regulate cell proliferation. This may be a factor, which leads to the upregulation of developmental genes like *PAX6*. This may lead to further upregulation of other TFs like *USF1* and *NF-E2*. All these TFs together may be acting in a concerted effort to bring about proliferation, transformation and vascularization.

E2F1 showed negligible change in mRNA expression between tumor and normal. It has been known that *E2F1* by means of Apaf-1 and p73 can activate caspases, which in turn lead to apoptosis (Tsantoulis and Gorgoulis 2005). This sheds light into the minimal expression of *E2F1* in cancer. If *E2F1* helps in the induction of apoptosis a cancer cell would be predicted to inhibit the activity of *E2F1* so that the cell does not undergo apoptosis. This would generate a tumor cell geared toward cell proliferation and transformation.

In a similar way *NKX2-5* showed a minimal change in expression between tumor and normal. Not much has been reported on *NKX2-5* in CRC. Finally, the dysregulation of the transcriptional regulatory network could lead to more cell proliferation, angiogenesis, malignant transformation, and metastasis to other organs like liver, lungs, and brain.

The analysis of wild-type survivin by Q-PCR showed an increase in gene expression within the same group of patient samples. This positively correlates with the increase in expression of candidate TFs that are being investigated for a role in the regulation of survivin and its expression-correlated genes. It can be proposed that upregulation of the panel of TFs eventually leads to the upregulation of survivin and its expression-correlated genes.

All of the data obtained by my analysis of TFs at the mRNA level indicate that increased expression occurs for many of the specific TFs that are involved in regulating mitosis and cell-cycle-progression. Specific TFs like *TP53* that become mutated or degraded blocks activation of its downstream protein $p21^{CIP}$, which is involved in cell-cycle damage checkpoint. This blockage promotes the passage of the cell through unregulated cell-cycles which leads to proliferation and tumor formation. Over-expression of specific TFs like *PAX6* and *MYB* contributes to cell-cycle dysregulation, which leads to more cell proliferation and oncogenesis. My mRNA analysis is one of the first steps in understanding how dysregulated expression of specific TFs leads to over expression of survivin and its expression-correlated genes in colon cancer compared to normal tissue.

The next step will be to analyze whether the increased expression of the candidate TFs at the mRNA level is reflected at the protein level in the malignant tissue compared to the adjacent normal tissue.

Chapter 3

DETERMINING THE PRESENCE OF CANDIDATE TRANSCRIPTION FACTORS AT THE PROTEIN LEVEL IN NORMAL AND MALIGNANT COLON TISSUE

3.1 Introduction

The IAP family of proteins is known to prevent programmed cell death and be involved in other cellular functions. Survivin, is the smallest member of the IAP family, and is thought when over-expressed, to be involved in cancer development including CRC. It is thought that the malignant transformation to CRC is largely due to the dysregulated gene expression induced by activation of *TCF*/β-Catenin signaling (Polakis 2000). Survivin, which is a downstream protein of *TCF-4*/β-Catenin, is predictive of decreased survival and increased tumor recurrence in patients (Sarela, Macadam et al. 2000). Based on large network analysis, I found that several other genes are expression-correlated along with survivin. Identifying mechanisms that regulate survivin and the expression-correlated genes, could pinpoint ways to target these regulatory mechanisms, which might help advance treatment and prevention strategies for CRC. To realize that possibility, it becomes critical that we find the TFs involved in the regulation of these genes. A first step is to determine which TFs are differentially expressed between normal and malignant colorectal tissues.

Cancer is associated with dysregulation of cell division that allows disruption of cell-cycle check points that in turn lead to increased proliferation of immature cells. In CRC, the canonical Wnt signaling pathway is activated by the mutation of the *APC* gene.

This renders *APC* inactive, which leads to constitutive expression of the β-catenin gene and then the increased expression of survivin and activation of its downstream targets.

Survivin is expressed at a basal level in normal crypt and usually localizes to the bottom portion of the crypt (Gianani, Jarboe et al. 2001) where proliferative cells reside. Survivin has been shown to be overexpressed in many tumors, compared with the adjacent normal tissue (Li, Ambrosini et al. 1998). It also has been used as a molecular biomarker to predict the aggressiveness of cancer (Altieri 2003). In human transcriptome analysis, survivin was one of the genes that was uniformly elevated in all types of cancer (Velculescu, Madden et al. 1999). All of these data point toward the premise that during tumor development, both the signaling and the transcriptional networks that regulate cell growth become dysregulated, which leads to a proliferative cell phenotype and an invasive histopathology. This suggests that the TFs that regulate survivin, as well as its expression-correlated genes, may play a role in up-regulating the expression of proliferative genes and lead to tumor development, and later CRC.

In cancer, oncogenic activation of TFs is a prominent event, which leads to mitotic and cell-cycle disruption, apoptotic inhibition, and acquisition of proliferative, invasive, and angiogenic properties. There are many families of TFs that play a central role in transformation of normal tissue into a cancerous, malignant, and invasive tissue. Below, I discuss the role that our identified candidates TFs have in colon tumorigenesis.

Rb is a transcriptional regulator that acts as an inhibitor of the *E2F*/DP family of proteins, which regulate expression of genes involved in cell proliferation and survival. *Rb* also promotes differentiation (Zhu 2005). *Rb* is one of the first genes found to become mutated in cancer development. Mutation of *Rb* leads to loss of its inhibitory effect on *E2F* family of TFs. Accordingly members of the *E2F* family, which have a tumor-

suppressor function, become deactivated, while those with proliferative functions are up-regulated.

Another major family of proteins involved in cell proliferation, differentiation, apoptosis, and transformation is the ETS groups of TFs. Twenty-seven members of this family have been recorded to date, one of which is *ELK1*. ETS proteins form complexes with many other TFs and enhance their transcriptional activity or regulate it in a target gene-specific manner. The ETS family has been found to regulate, positively or negatively, expression of genes involved in tumor formation and cell proliferation. In CRC, there is high expression of ETS family members at the advanced stage of the disease. Expression of ETS1 and ETS2 are at a basal level in normal and hyperplastic colon, but in CRC can serve as a marker for the tumor stage and correlate with lymph node metastasis (Ito, Takeda et al. 2002). ETS1 also correlates with integrin beta-3 expression during lung metastasis from CRC, illuminating a role for ETS1 and its target gene in transformation and invasion of stromal cells. Hence, from these observations it can be proposed that *ELK1* might play a role in CRC formation.

Another master TF gene, *TP53*, has been shown to regulate numerous other TFs and proteins. But p53 becomes mutated or inactivated in most cases of cancer. This leads to aberrant transcriptional regulation, which alters the expression of the downstream target genes (Menendez, Inga et al. 2006). There are transcriptional regulatory switching mechanisms in cancer, compared with normal tissue, such that many of the tumor-suppressor genes become inactivated or mutated and oncogenic genes become activated. Hence, evaluating the differential expression of the candidate TFs that are proposed to regulate survivin and its expression-correlated genes will provide insight into how changes occur in transcriptional regulation during CRC development.

Once we have identified which of the TFs among the panel of candidate TFs are expressed differentially at the mRNA level, the next logical step is to examine whether the expression occurs at the translational level. There can be instances where the translation of mRNA is inhibited which leads to decreased protein production. Using two independent experimental approaches (i.e. analysis of mRNA and protein) reduces the chance that any artifacts have arisen. Hence, it becomes essential to examine whether differential expression of individual TFs at the mRNA level correlates with the differential expression at the protein level. This leads to my next immediate hypothesis: *Specific TFs in the set of candidate TFs are expressed differentially at the protein level in normal and cancerous colonic mucos*a.

3.2 Materials and Methods

3.2.1 Patient Tissue Slides

All matched normal and CRC tissue sample slides were prepared at Christiana Hospital after patient consent and I*RB* approval. The tissues were fixed in 10% formalin for 2 hours and later passed through an increasing alcohol series to rehydrate, then subjected to xylene. Later, the tissues were embedded in paraffin and paraffin tissue blocks were sliced into 5-micron sections at the pathology department of Christiana Hospital. Our lab was given the 5-micron formalin-fixed, paraffin-embedded tissue sections.

3.2.2 Immunohistochemistry (IHC)

Immunohistochemical staining was performed on 5-micron, paraffin-embedded tissue sections. The slides were deparaffinized using xylene and later subjected to rehydration using a series of ethanol. Antigen retrieval was performed using Target Antigen Retrieval Agent (Dako) in a microwave at maximum power for 25 seconds and later at a reduced power for 12 minutes. After cooling, the sections were washed in 1x

Tris buffered saline (TBS). The endogenous peroxidase activity was quenched using 3% hydrogen peroxide in methanol for 10 minutes. Later, the tissue sections were blocked using a serum block in a humid chamber and incubated with appropriate concentration of primary antibody (Table 6) at 4°C overnight. After appropriate washing in 1xTBS, the tissue sections were treated using the Zymed Histostain Plus kit, which includes a broad-spectrum biotylinated secondary antibody, which is later incubated with a streptavidin conjugated horse-radish peroxidase enzyme. The color was developed using a DAB (3, 3'-diaminobenzidine tetrahydrochloride) kit (Zymed). After washing the tissue sections were counterstained using Harris haematoxylin for 2 minutes, washed, and completely air-dried. Later sections were mounted using DPX mountant for histology and coverslipped. Negative controls including the absence of primary antibody were used to evaluate the specificity of the reactions. Final images were taken at an objective magnification of 40X. Scale bars for 15µm were included in the images.

Table 6: Primary antibodies used for Immunohistochemistry

Antigen	Source	Dilution
TP53	(DO-1)-mouse IgG_{2a} (Santa Cruz Biotechnology, CA)	1:100
MYB	(SEQer)-Rabbit IgG (SDIX, DE)	1:250
E2F4	(C-20)-rabbit IgG (Santa Cruz Biotechnology, CA)	1:50
E2F1	(KH-95)-mouse IgG_{2a} (Santa Cruz Biotechnology, CA)	1:50
PAX6	AB5409-Rabbit-IgG (Chemicon, CA)	1:100

3.2.3 Immunofluorescence (IF)

Paraffin tissue sections 5 microns thick were deparaffinized using xylene and later rehydrated using an alcohol series. Antigen retrieval was performed using Target antigen retrieval reagent (Dako) in a microwave. After cooling, the tissue sections were washed in 1x TBS for 5 minutes three times and incubated with 1% BSA in 1xPBS for 90minutes. Without rinsing, the tissue sections were incubated with primary antibody (*NKX2-5* (N-19), Goat Polyclonal IgG (Santa Cruz Biotechnology, CA)) in a humid chamber at 4°C overnight. After incubation, the tissue sections were washed with 1xTBS three times for 5 minutes. Then the sections were incubated with Alexa Fluor 488 conjugated secondary antibody for 1 hour. After washing with 1xTBS, three times for 5 minutes each, the tissues sections were mounted with Slowfade Gold Antifade with DAPI (Invitrogen) along with nuclear stain Draq5 (Biostatus), coverslipped, and kept at -20°C in the dark until viewing. Tissue slides without primary antibody were used as negative controls.

In the Immunofluorescence (IF) micrographs, the green stain is the primary antibody against *NKX2-5* using a FITC configured filter with a wavelength of light at 488nm. The blue is nuclear stain for Draq5, with a Cy5-configured filter using a light of wavelength 633nm. Images for normal tissue were taken at an objective magnification of 20X, to capture full-length crypts. Tumor tissue was taken at a magnification of 40X, because the tissue is disorganized without visible crypt structure. Scale bars for 50μm for normal tissues and *20um* for tumor tissues were included in the images.

3.3 Results

3.3.1 Immunohistochemistry

IHC staining was done to analyze the expression of five of the candidate TFs including p53, *MYB*, *E2F4*, *E2F1* and *PAX6*. As the antibody available for *NKX2-5* works only for IF not IHC, IF was done for *NKX2-5*.

The IHC results on P53 showed no staining for normal mucosa. But in cancer tissues 90% of samples stained strongly positive with staining confined to the nucleus of the malignant epithelial cells (Figure 11). The tumor stroma showed no staining. There were some tumors that showed a heterogenous staining pattern for *TP53*.

MYB showed positive nuclear staining in normal tissue, positively staining cells were located more in the proliferative and upper regions of the colonic crypt, with minimal staining at the basal region. Compared with normal tissues, tumor tissues showed very intense nuclear staining for *MYB* with no staining of the stromal cells (Figure 12).

IHC for *E2F4* gave positive nuclear staining of cells in normal as well as the tumor tissues. In normal tissue, the staining pattern was generally uniform throughout the crypt with occasional cells staining negative for *E2F4*. In tumor tissues there was positive nuclear staining of cells as well, but staining for *E2F4* was not as uniform (Figure 13). The stromal cells showed minimal or no staining. In the case of the other *E2F* family member *E2F1*, results showed only a few cells staining positively in normal tissue and no staining in the tumor tissue. Specifically, there were isolated cells showing nuclear staining for *E2F1*, these, were localized to the proliferative crypt region, but no staining was seen in the tumor tissues (Figure 14).

The paired box protein, *PAX6*, showed nominal staining of cells in normal tissue and intense nuclear staining in the tumor tissues (Figure 15). The intensity of the staining was much stronger in the tumor tissue sections compared with the normal slides. The negative controls for IHC involving absence of primary antibody did not show any staining in normal or tumor tissue sections (Figure 16).). Scale bars for 15µm were included in the IHC slides.

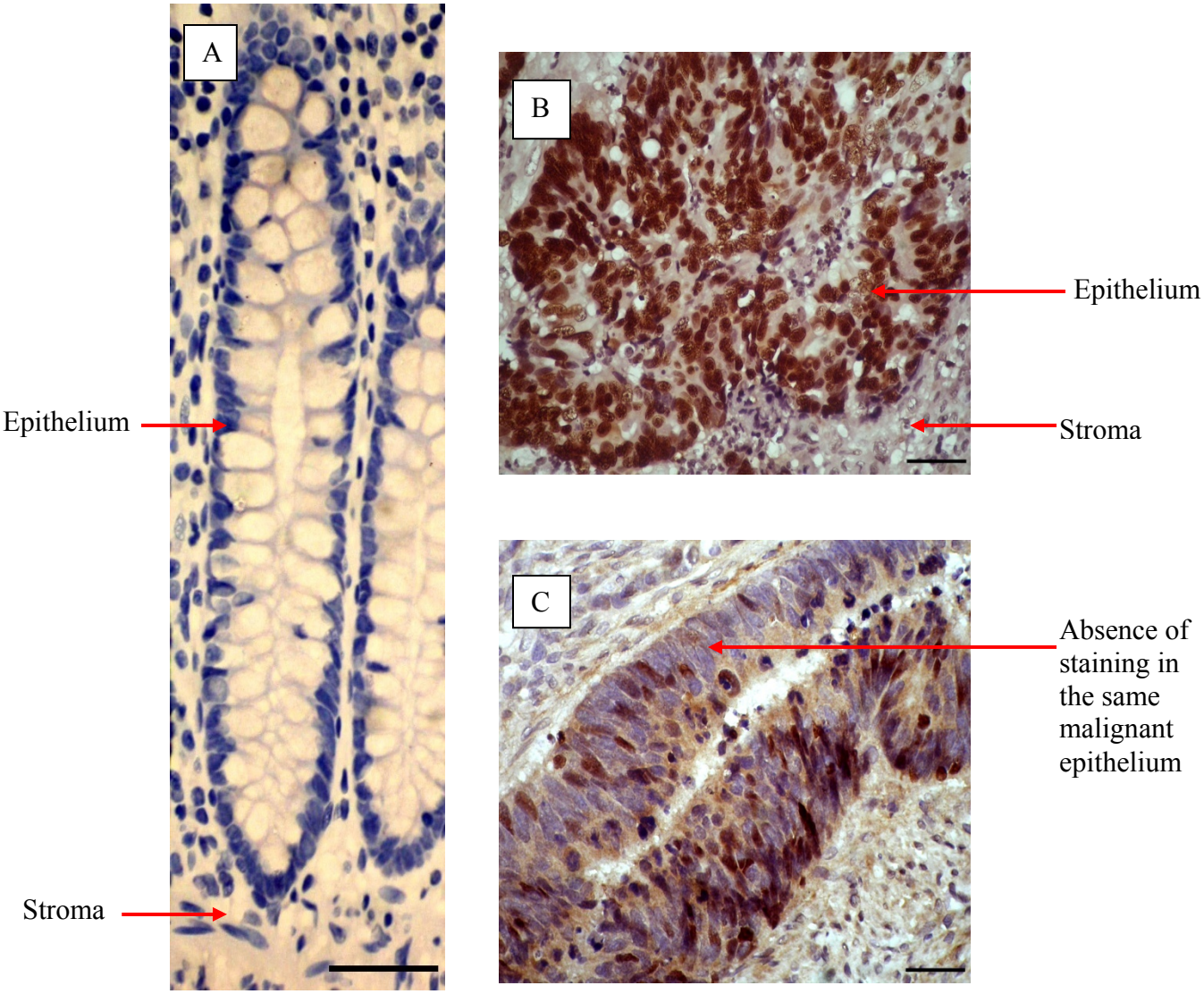

Figure 11: Immunohistochemical staining for p53. Normal tissue (Patient# 7074) showed minimal to absence of expression for p53 in the colonic epithelium as well as stroma (A). Tumor tissue (Patient # -TB09) showed high expression of p53 which was localized to the nucleus (B). But some tumor tissues in the same sample (Patient #-7181) or in the same malignant epithelium itself had some cells showing intense nuclear staining while other cells showed negative staining (C). Scale bars represent 15µm.

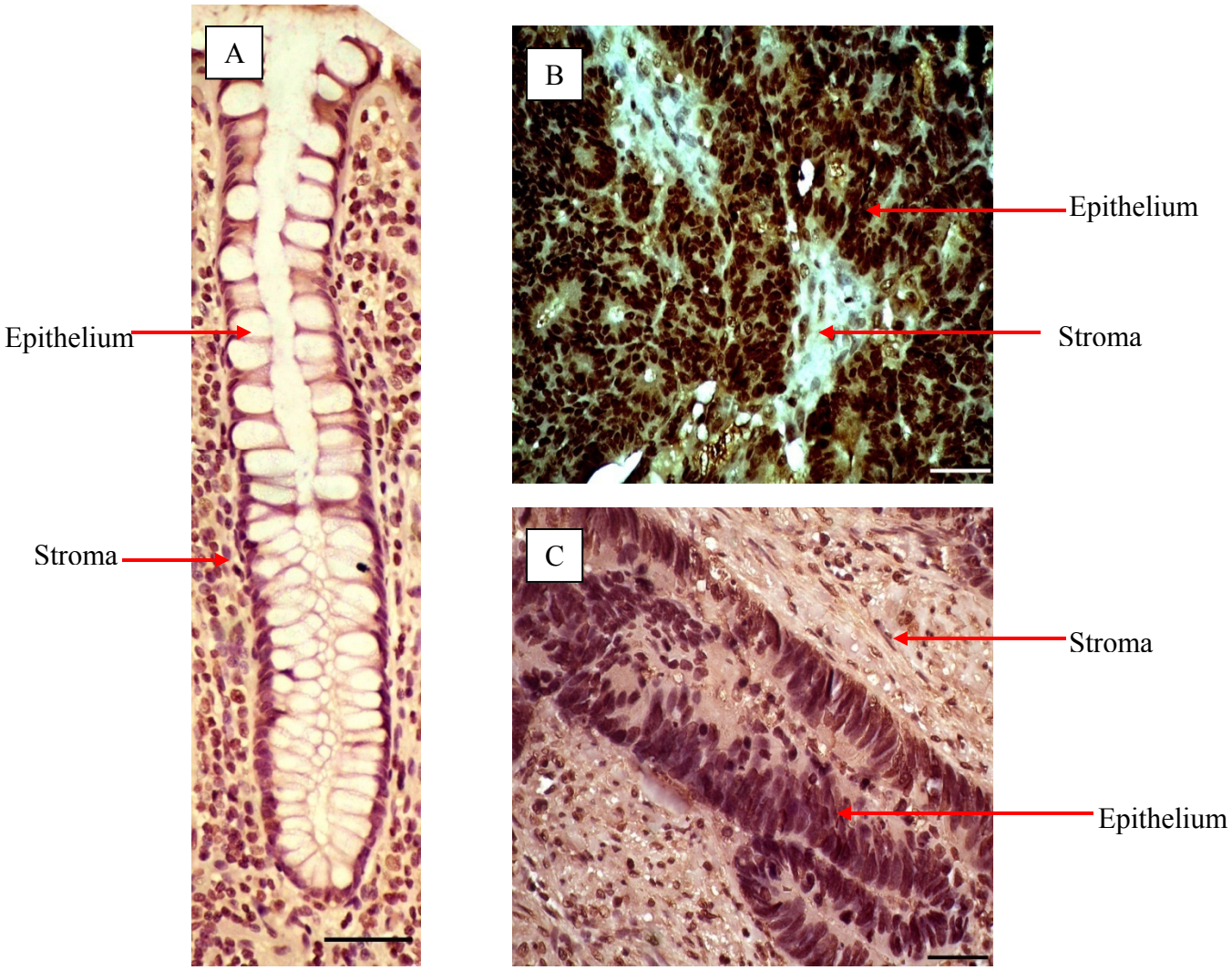

Figure 12: Immunohistochemical staining for *MYB*. Staining for *MYB* showed positive nuclear staining in normal tissue (Patient #-7074) as well as the tumor tissue. Normal tissue had positively staining cells throughout the crypt (A). Most tumor tissues had intense staining of the carcinoma cells (Patient #-7181) with minimal staining of the stroma (B). Even though some tissues (Patient #-7073) showed positive nuclear staining in some parts of malignant epithelium, there were other parts that showed partial positive staining (C). Scale bars represent 15µm.

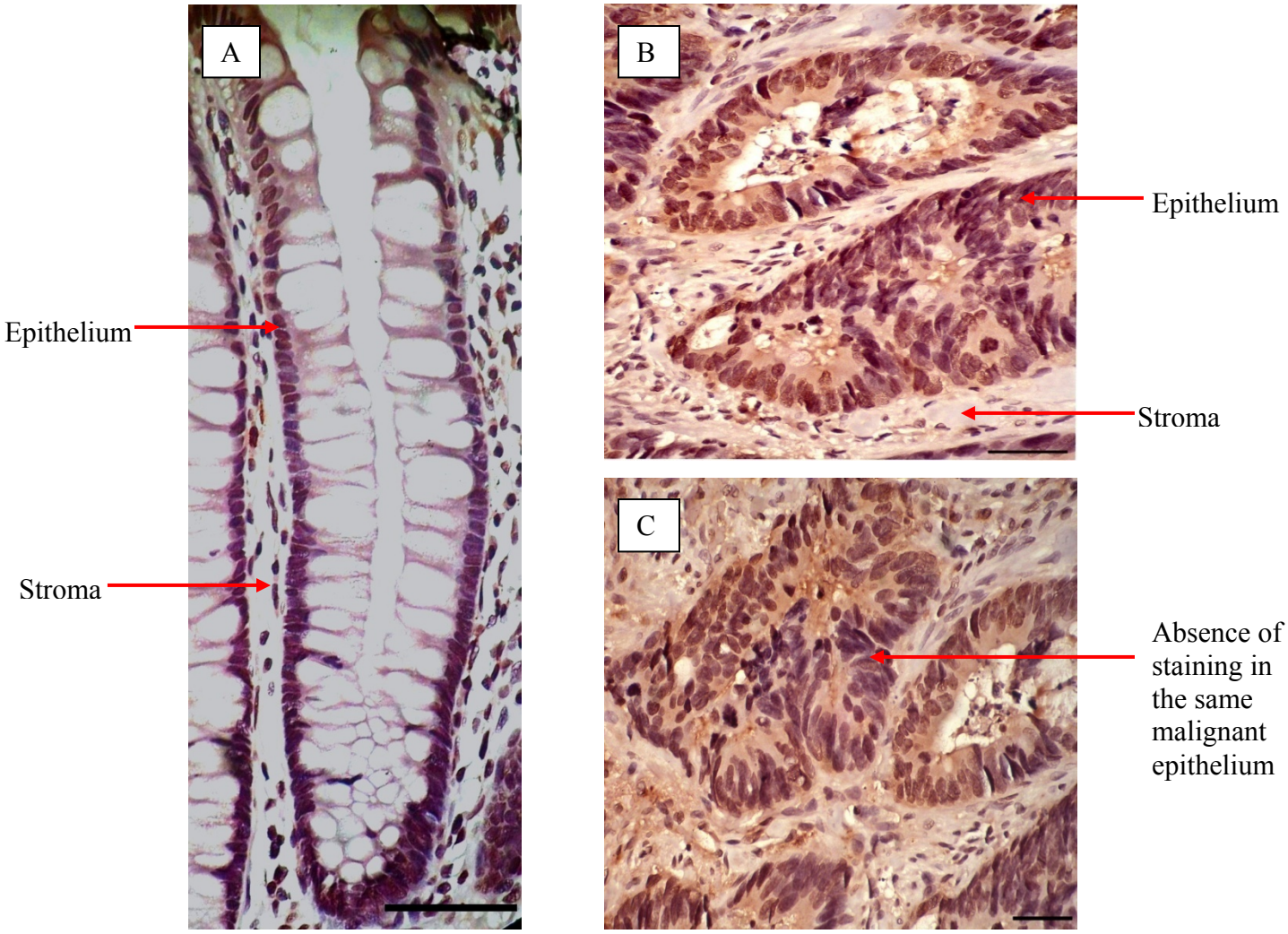

Figure 13: Immunohistochemical staining for *E2F4*. Staining for *E2F4* showed positive nuclear staining in normal tissue as well as the tumor tissue. Normal tissue (Patient #-7077) had positively staining cells throughout the crypt (A). Tumor tissues had intense nuclear staining of the carcinoma cells (Patient #-7073) with some staining of the stroma (B). Even though some tissues showed positive nuclear staining in some parts of malignant epithelium (Patient #-7073), there were other parts of the same epithelium that showed no nuclear staining (C).Scale bars represent 15µm.

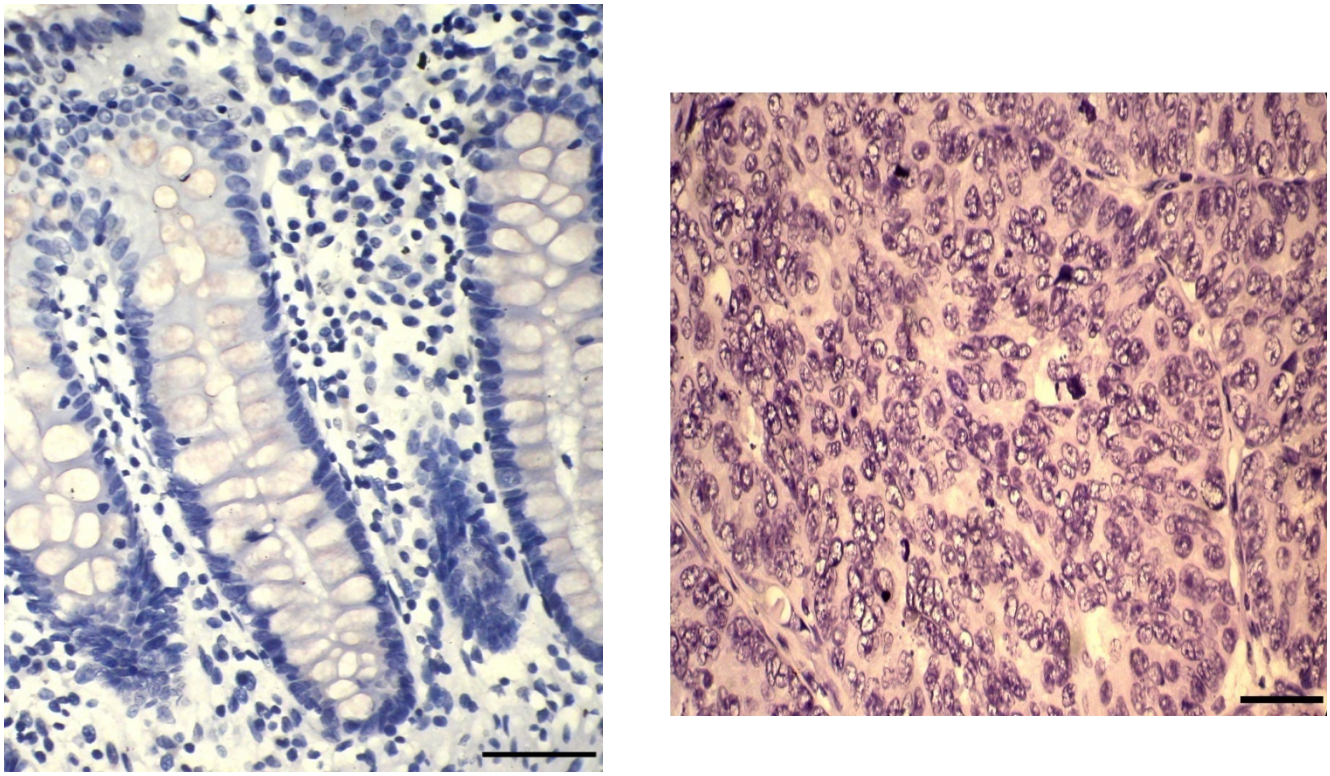

Figure 14: Immunohistochemical staining for *E2F1*. Normal tissue is in the left panel and malignant colon tissue in the right. There was no staining for *E2F1* in normal tissue or in the tumor tissue. There were isolated cells staining positive for *E2F1*, which were localized to the proliferative region of crypts in some of the tissue sections (data not shown). There was no staining in the tumor tissues. Scale bars represent 15μm.

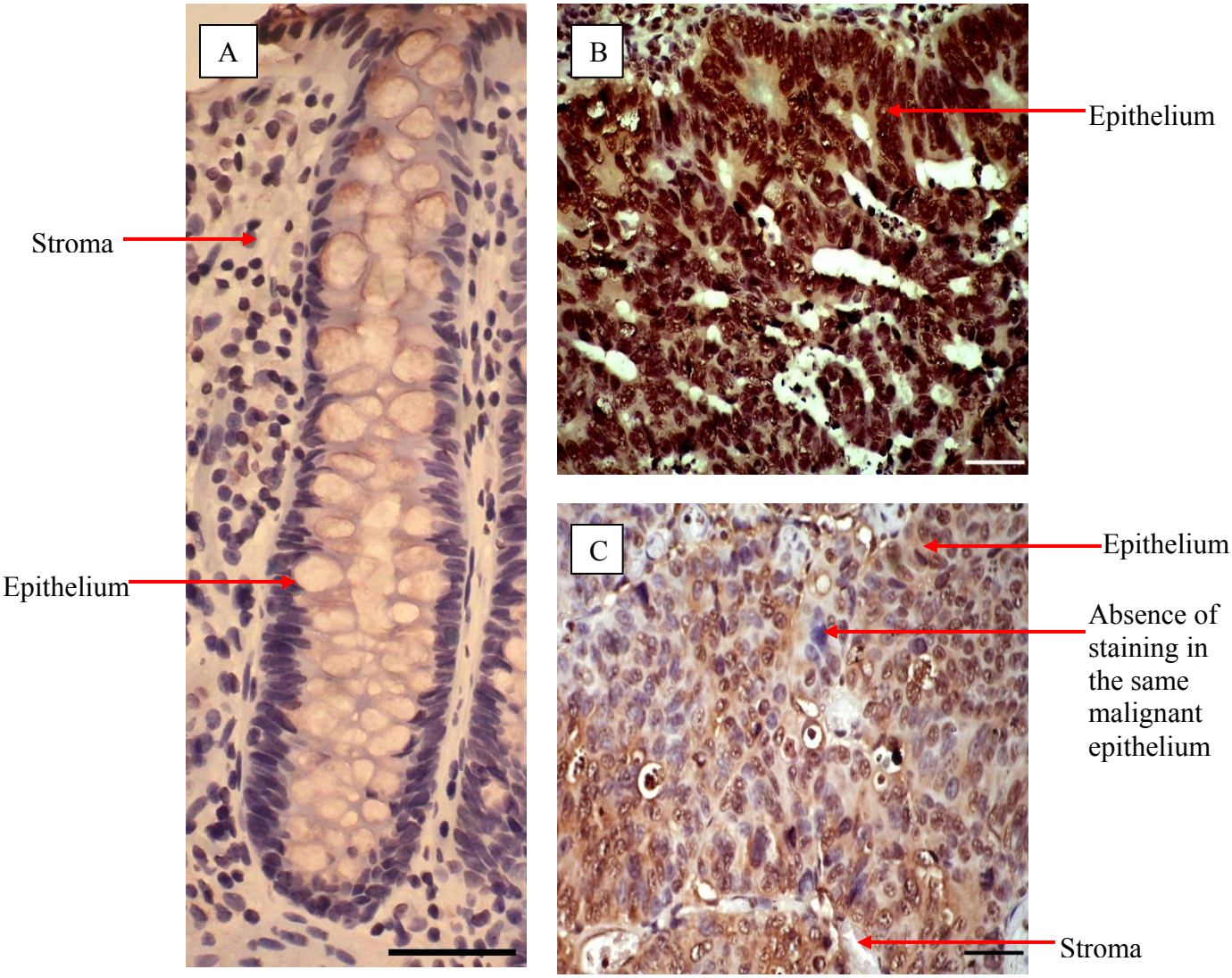

Figure 15: IHC staining for the TF *PAX6*. Staining for *PAX6* showed absence of nuclear staining (A) in normal tissue (Patient #-7074) while some tumor tissues (Patient #-TB05-41) gave intense nuclear staining (B). There were tumor tissues (Patient #-7206) where there was epithelium, which had cells that showed positive nuclear staining while other cells showed absence of nuclear staining (C). Scale bars represent 15µm.

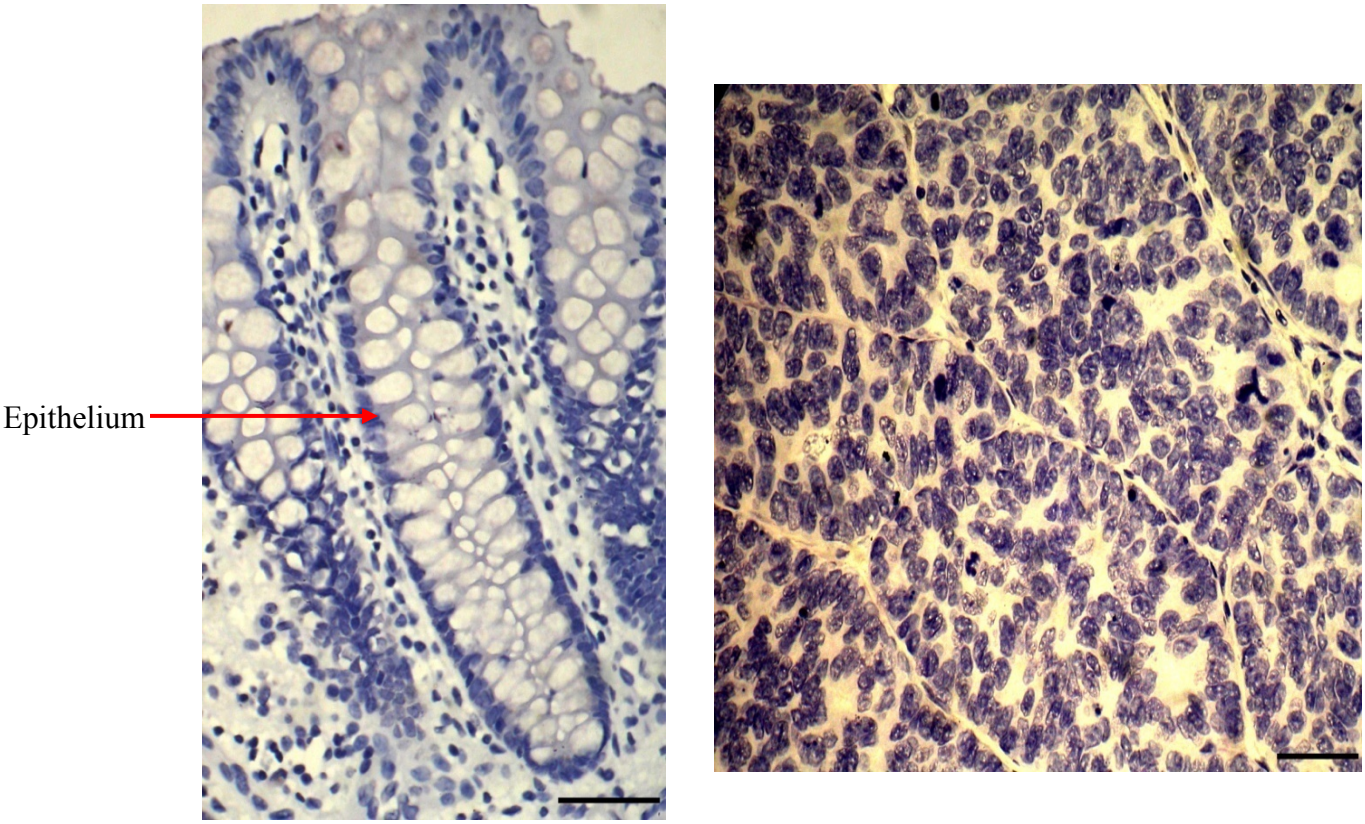

Figure 16: Negative control for IHC. Tissue sections, that excluded primary antibody for normal tissue (left) and carcinoma tissue (right). There was no staining of cells in normal or tumor tissue sections. Scale bars represent 15µm.

3.3.2 Immunofluorescence (IF)

The IF staining of normal tissue for *NKX2-5* didn't give any specific staining of crypt cells, but there was faint non-specific staining at the top portion of the crypt localized within the cytoplasm (Figure 17). In tumor tissue there was very bright positive fluorescent staining localized to the cytoplasm of the carcinoma tissue (Figure 18). No staining was observed in the adjacent stromal tissue. The negative control in the absence of primary antibody for normal tissue (Figure 19) showed no staining for the colonic crypt and the negative control for tumor (Figure 20) showed no staining of the carcinoma cells.

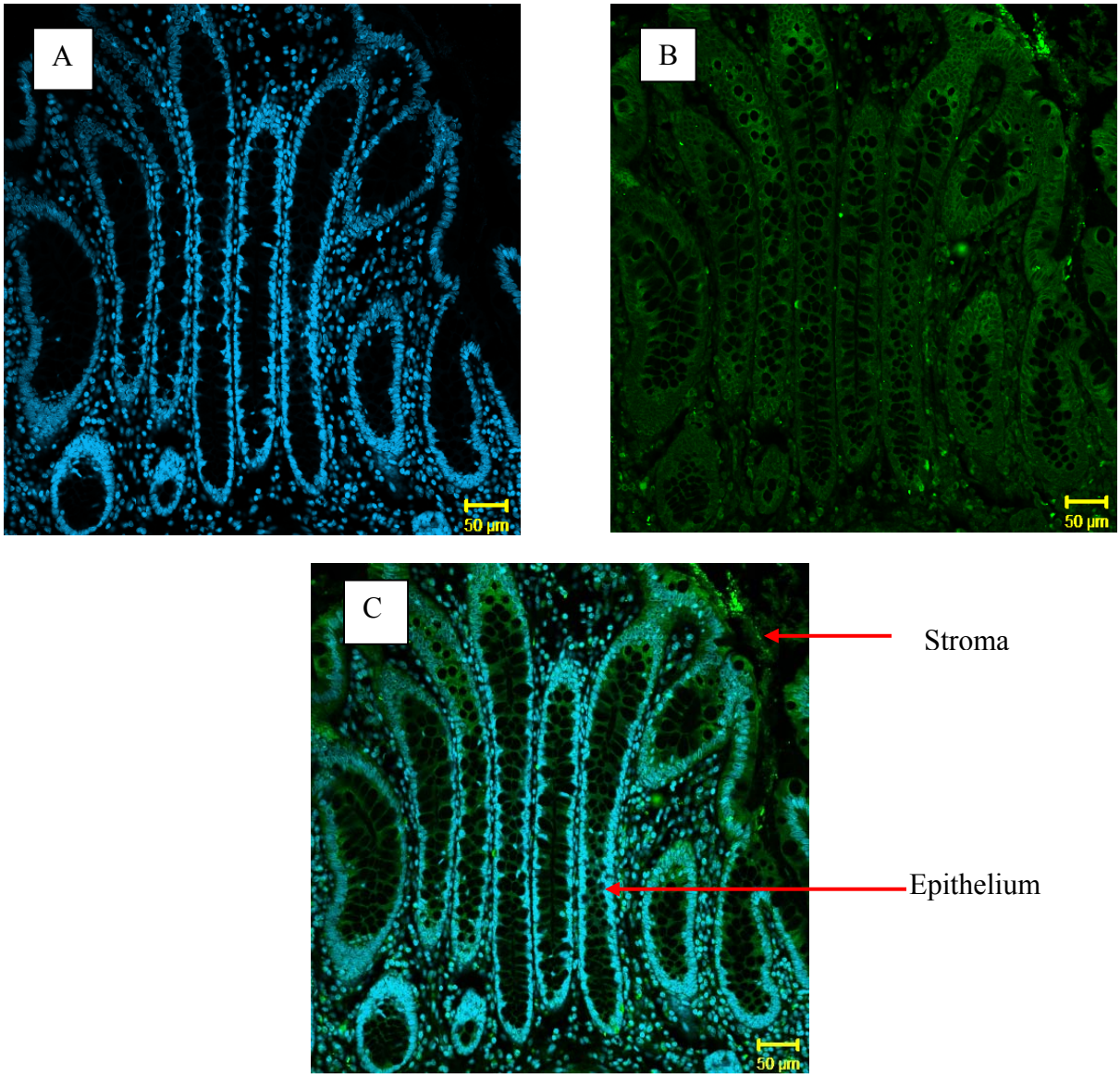

Figure 17: Immunofluorescence staining for NKX2-5 in normal colonic tissue. There were one or two cells showing positive nuclear staining in the bottom part of the crypt, otherwise the crypt showed absence of staining for NKX2-5. Stroma showed some staining. Panel A shows nuclear staining (blue), panel B shows staining for NKX2-5 (green) and panel C shows merged image.

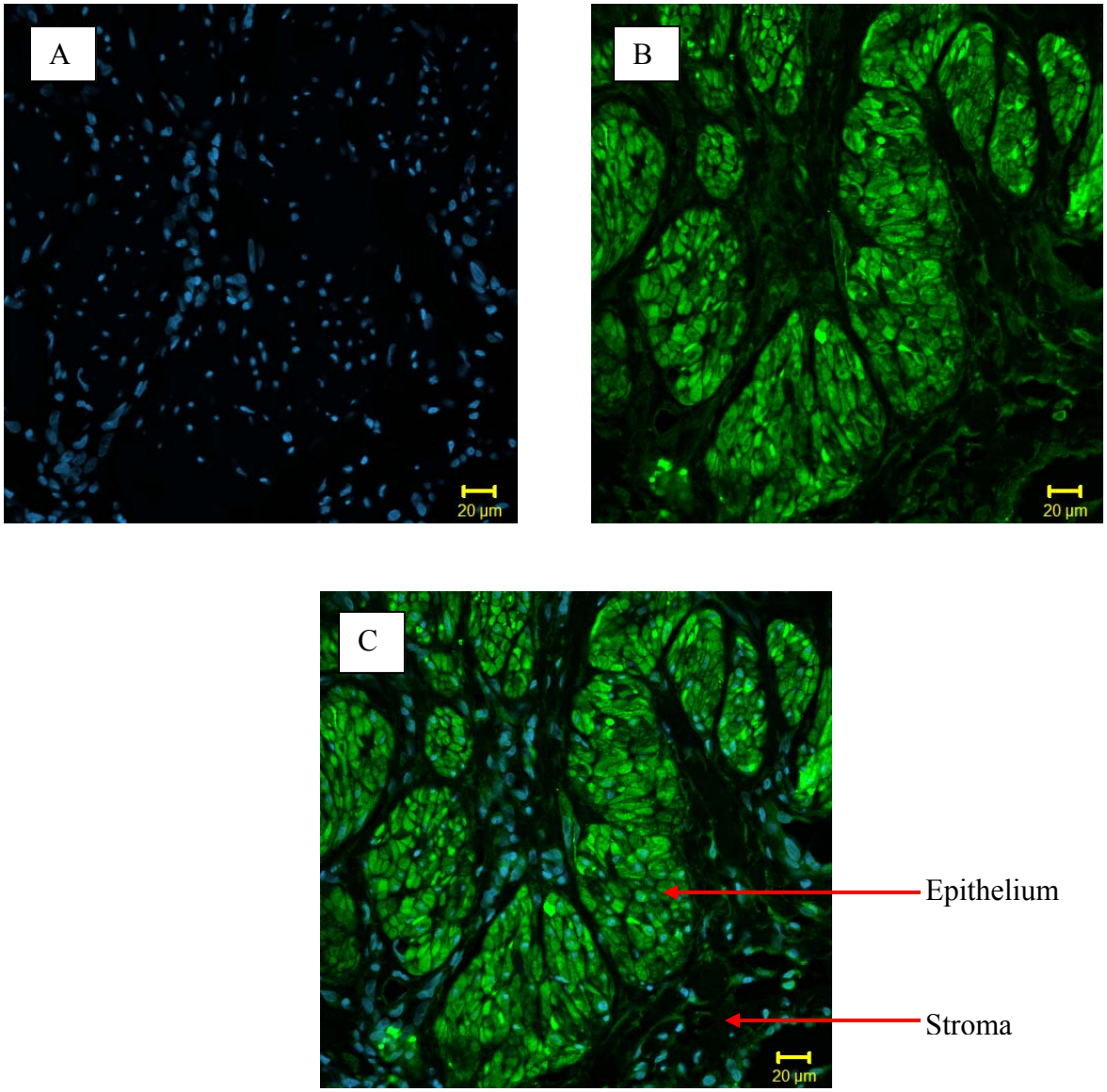

Figure 18: Immunofluorescence staining for *NKX2-5* in tumor tissue. Tumor tissue showed intense staining mostly localized to epithelial cytoplasm even though some nuclear staining was visible. Stroma showed minimal staining compared to the epithelium. Panel A shows nuclear staining (blue), panel B shows staining for *NKX2-5* (green) and panel C shows merged image.

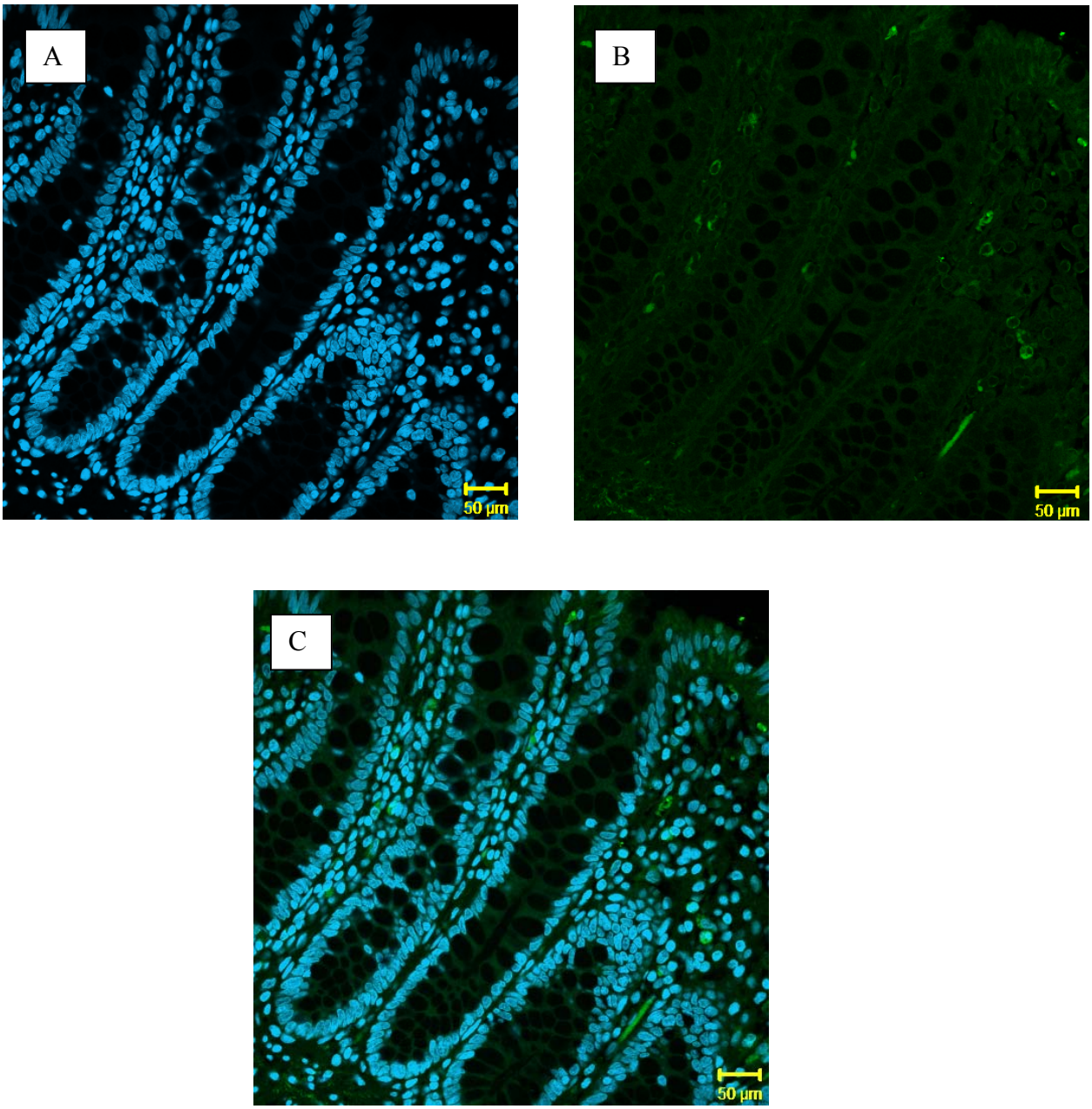

Figure 19: Negative control staining (IF) in the absence of primary antibody for normal tissue. There was absence of staining in the colonic epithelial cells. Panel A shows nuclear staining (blue), panel B shows staining for *NKX2-5* (green) and panel C shows merged image.

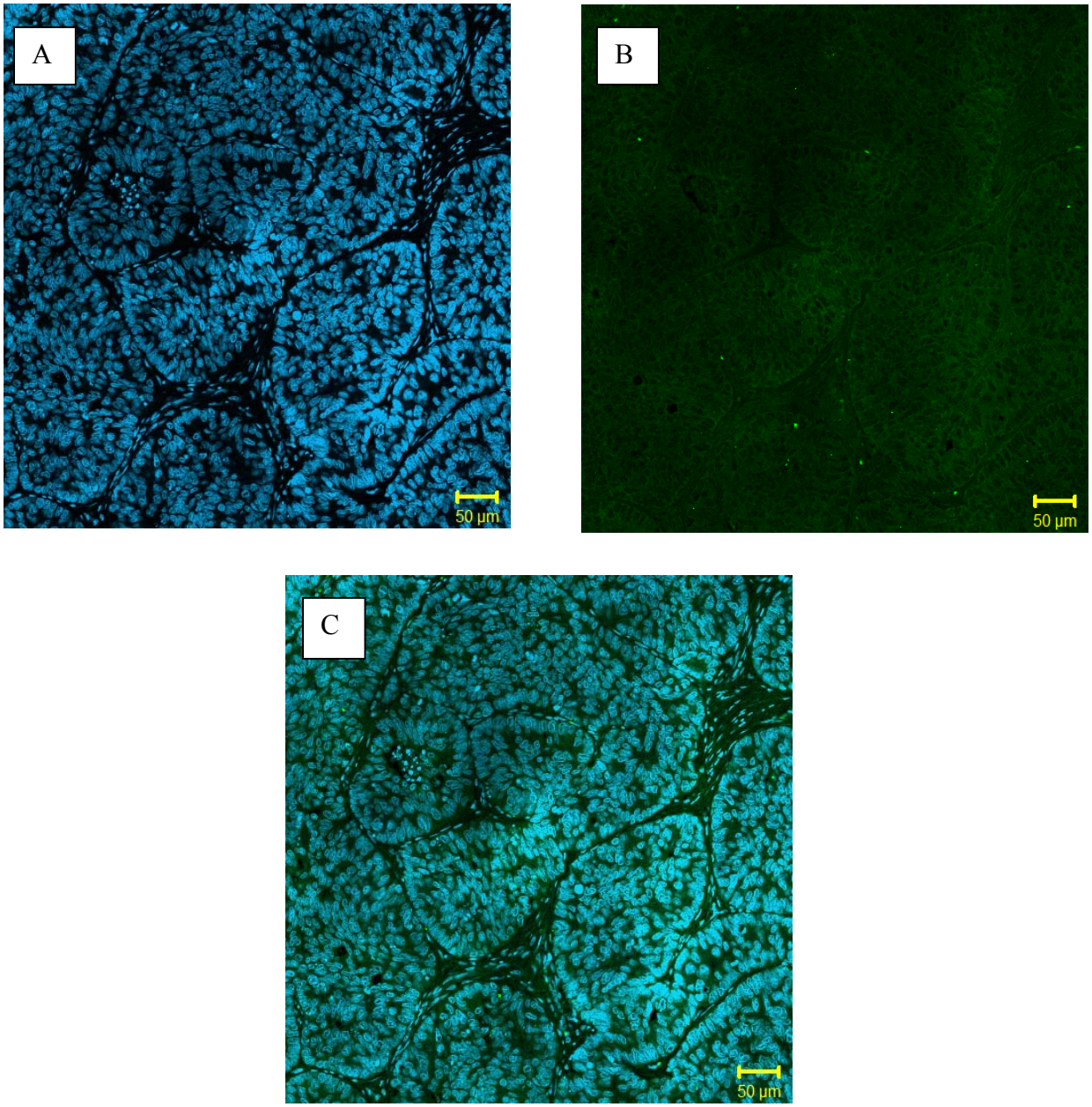

Figure 20: Negative control staining (IF) in the absence of primary antibody for tumor tissue. There was absence of staining for *NKX2-5* in the carcinoma cells. Panel A shows nuclear staining (blue), panel B shows staining for *NKX2-5* (green) and panel C shows merged image.

3.4 Discussion

Using state-of-the art correlation analysis of multiple experimental gene expression platforms, we showed that there is a set of genes that is expression-correlated along with the survivin gene (*BIRC 5*). It was predicted that the expression of this set of genes may be co-regulated by a set of TFs. From PAINT analysis for over-represented TREs in promoter regions of this gene set, a panel of candidate TFs was selected that consisted of TFs involved in cell-cycle and regulation. To examine whether TFs are differentially expressed at the protein level in CRC versus normal colonic mucosa, protein expression studies were performed using IHC and IF.

TP53 was one of the genes in the panel of specific TFs that we analyzed. P53 is an important protein involved in various physiological aspects including tumor suppression, cell-cycle regulation and apoptosis. It is mutated in as many as 50% of cancers, including CRC cases. Cancer driven mutations in *TP53* are predominantly point mutations that result in single amino-acid substitutions. The mutant proteins are often more stable than wild-type p53, and are present at very high levels in cancer (Vousden and Lu 2002). This may be because mutant p53 protein act as dominant negative that inhibits wild type p53 protein (de Vries, Flores et al. 2002). In many instances where *TP53* is not mutated in cancers, it becomes inactivated. In p53-null mice, which are highly susceptible to the early development of cancer, the tumor suppressor capability of transfected wild-type *TP53* was demonstrated (Donehower, Harvey et al. 1992). P53 is a master TF that affects the function of many other regulatory molecules including transcription factors such as the E2F family. The E2F family by itself plays a pivotal role in cell fate and particularly in cancer evolution. By virtue of their innate ability as well as their interaction with *TP53*, the E2F family contributes to the oncogenic stimulus that leads to full-fledged cancer (Polager and Ginsberg 2009).

TP53 also acts on many other genes like survivin (Hoffman, Biade et al. 2002) by virtue of its binding to the target response elements (REs) in their promoter regions (Riley, Sontag et al. 2008). Moreover, p53 interacts with another protein *NFY*, (Benatti, Basile et al. 2008) which is one of the genes included in our panel of TFs. In human colon adenocarcinoma cell lines, upregulation of Annexin-A1 was induced by butyrate, which was found to be mediated by a release of NF-Y from a proximal CCAAT box and an enhancement of p53 binding (Lecona, Barrasa et al. 2008). My IHC analysis for p53 showed minimal to no staining in most normal tissues and intense positive staining localized to the nucleus of cells in most of the tumor tissues analyzed (Figure 8).. This relates to the fact that in some cancer cases p53 is mutated, where it results in positive staining even though the protein has lost its function. But some tumor tissue samples didn't show any positive staining for p53,. This may be because in those particular cancers wild type p53 protein is lost or degraded, leaving no detectable p53, which can be analyzed via staining.

I also analyzed *E2F4* and *E2F1*. These two TFs are in the *E2F* family, which includes eight members. *E2F1* is a transcriptional activator that modulates quiescent cell population through transition into S-phase of the cell-cycle. *E2F1* possesses the ability to induce p53-dependent and p53-independent apoptosis, a function related to oncosuppression (Tsantoulis and Gorgoulis 2005). In contrast *E2F4* is usually upregulated in colon cancer and inhibits apoptosis (Mady, Hasso et al. 2002). These findings are consistent with our IHC staining. *E2F1* showed minimal staining in the normal colonic crypt, with only a few cells showing staining in the proliferative part of the colon. In tumor tissue, there was no staining. This staining pattern correlates with the fact, discussed above, that *E2F1* is an oncosuppressor and, in CRC, is down-regulated, allowing cancer cells to proliferate unchecked. In contrast, our staining for *E2F4* gave a

positive nuclear staining pattern in normal tissue, with cells staining uniformly throughout the crypt. In malignant tissues, *E2F4* gave an intense staining localized to the nucleus in some cells of the colonic epithelium, while other cells stained less intensely. This again correlates with the literature, in that *E2F4* tends to be up-regulated in CRC, which inhibits apoptosis promoting proliferation and tumorigenesis.

MYB is another TF in the panel that we tested, one that is known to exhibit increased expression in cancers. In patients with breast cancer, *MYB* over-expression is associated with poor prognosis (Amatschek, Koenig et al. 2004). *MYB* is known to be involved in growth of hematopoietic leukemias and some epithelial cancers. *MYB* expression has also been shown to be increased in colon carcinomas and in adenomatous polyps, compared with normal mucosa (Ramsay, Thompson et al. 1992). *MYB* contributes to malignant transformation as demonstrated by its ability to repress differentiation in differentiation-prone cell lines like colonic epithelial cell lines (Thompson, Rosenthal et al. 1998). According to our IHC analysis, staining for *MYB* gave positive nuclear staining throughout the normal colonic crypt. In malignant tissue, staining for *MYB* showed very intense staining localized to the nucleus, suggesting that *MYB* is highly upregulated in tumor tissue, compared with the normal**.** This suggests that *MYB* has a major role in oncogenic transformation in the colon.

I also evaluated *PAX6*, a TF that functions in the development of the eye, other sense organs, and certain neural and epidermal tissues. It is included in a family of paired box genes consisting of nine family members. *PAX* genes also have been implicated in the development of cancer. In a cancer-wide analysis of *PAX* gene expression, it was seen that *PAX* genes were expressed constitutively even though the expression pattern did not match p53 status or other cancer-related genes like *BCL-2* (Muratovska, Zhou et al. 2003). My IHC analysis for *PAX6* expression in normal tissues

showed a nuclear staining pattern involving the bottom half of the crypt. There was positive staining of the cells in the basal part, where the SC are supposed to reside, as well as in the proliferative compartment. There was less staining in the top portion of the crypt. In carcinoma tissue, there was intense staining for *PAX6*. The stroma showed minimal staining for *PAX6*. This indicates that *PAX6* expression is up-regulated in cancer tissue and this may be contributing to the proliferative ability and prolonged survival of the cancer cell.

IF was used to analyze the TF *NKX2-5*. This is a major developmental gene involved in cardiogenesis during embryo formation. It has been shown that Wnt signaling via *β-catenin* is involved in cardiomyocyte differentiation and proliferation. *β-catenin* knockout mice were found to have deformed hearts with poorly developing cardiomyocytes and reduced proliferative potential in both ventricles (Kwon, Arnold et al. 2007). *NKX2-5* is a master regulatory TF that controls *β-catenin* expression during cardiogenesis. *NKX2-5* binding elements have been found in the promoter region of the *β-catenin* gene (Riazi, Takeuchi et al. 2009). *β-catenin* is upstream in the survivin signaling pathway.

NKX2-5 is a nucleophosphoprotein whose nuclear localization signal (NLS) needs basic amino-acids in its homeodomain to localize to the nucleus. If there is any mutation in the homeodomain the TF is unable to localize to the nucleus (Komuro and Izumo 1993). In our IHC analysis, *NKX2-5* was found to be highly expressed in the malignant tissue compared with the normal colonic epithelium. The normal tissue showed minimal staining by IF. The staining in the malignant tissue was intense and localized more to the cytoplasm. This indicates that *NKX2-5* is upregulated in CRC, compared with normal colonic epithelium. It can also be proposed that in cancer the homeodomain of NLS gets mutated so that the mutant protein is not able to be transported to the nucleus.

Based on these findings, it can be hypothesized that *NKX2-5* may be acting via *β-catenin* leading to dysregulation of the canonical Wnt signaling pathway that maintains colonic epithelial homeostasis as well as acting on survivin expression to promote mitosis and inhibit apoptosis. Angiogenesis also occurs during tumorigenesis. *NKX2-5* is also involved in blood vessel formation (Schwartz and Olson 1999), which may be playing a part in angiogenesis as well.

Of the six candidate TFs that were tested at the protein level using immunostaining, five of the factors (*TP53*, *MYB*, *E2F4*, *PAX6* and *NKX2-5*) gave a differential expression in tumor compared with the normal colonic tissue. These TFs showed increased expression in tumor tissue compared with normal tissue. *E2F1* showed no change in expression pattern tumor tissue compared to normal tissue. All these results correlate with my Q-RT-PCR analysis.

Taken together the Q-RT-PCR as well as the immunostaining data indicate that the panel of candidate TFs is expressed differentially in tumor, compared with the normal colonic epithelium. The increased expression of *E2F4*, *PAX6*, *MYB*, and *TP53* at both the mRNA and the protein levels, and NKX2-5 at the protein level, attest to the fact that increased expression occurs for many of the specific TFs that are involved in regulating mitosis and cell-cycle progression. The specific TF like *TP53* gets mutated or degraded which blocks activation of its downstream protein $p21^{CIP}$, which is involved in cell-cycle damage checkpoint. Its blockage allows the cell to pass through unregulated cell-cycles which leads to proliferation and tumor formation. Along with the unregulated cycles of cell-cycle there is over-expression of the specific TFs like *PAX6* and *MYB*, which contribute to more cell proliferation and oncogenesis. Still it is interesting to note that *NKX2-5* showed negligible change in expression at the mRNA level, but was observed to have high expression in the cytoplasm at the protein level. This may be due

to the possibility that *NKX2-5* mRNA is becoming degraded while the *NKX2-5* protein is modified post-translationally (Kasahara and Izumo 1999) so that it is more stable. Also it was noticed that even though *NKX2-5* positivity localized to nucleus, it was localized much more in the cytoplasm of the epithelial cells. It can be hypothesized that *NKX2-5* protein over-production could have caused mutation in the NLS (Komuro and Izumo 1993), so that instead of getting transported to the nucleus; the protein could have stayed in the cytoplasm. It was seen that some of the protein was localized to the nucleus, which could have gotten transported.

My results show that specific TFs like *TP53*, *MYB*, *NKX2-5* and *E2F4* are differentially expressed in CRC, compared with normal colonic epithelia. These are also the TFs that showed over-represented TREs in the promoter regions of survivin and its expression-correlated genes. Many of the TFs in the panel, like *E2F4*, regulate the process of cell-proliferation and cell-cycle. It is seen that these TFs are expressed at more than a fivefold increase in the mRNA level and very high expression in the protein level. Thus over-expression of the candidate TFs is consistent with our hypothesis that specific TFs lead to increased transcription and translation of survivin expression-correlated genes.

A delicate balance exists between signaling molecules involving TFs and other proteins present in normal tissue rendering homeostasis. In cancer, this balance is lost, which leads to increased expression of TFs involved with proliferation and suppression of TFs involved in apoptosis. This leads to uncontrolled proliferation and transformation of cells. The data from our study here suggest that cancer arises, in part, due to select TFs that are involved in cell-cycle progression (including mitosis) that become deregulated. This in turn, leads to a dysregulated transcriptional network, which might increase the expression of survivin and its expression-correlated genes. This

increased activity of the expression-correlated genes might have a role in the progression and development of CRC. This investigation is a first step to the understanding of transcriptional control mechanisms involved in the co-regulation of survivin and its expression-correlated genes.

Survivin itself has been implicated in mitosis as well as apoptosis (Altieri 2008). It has been demonstrated that survivin interacts with proteins such as p53 and *E2F* family members, which are major gate keepers of cell-cycle and proliferation and are dysregulated in cancer. This can lead to dysregulated cell proliferation and eventually cellular transformation to form cancer. It can be hypothesized that survivin expression-correlated genes are highly over-expressed in CRC due to the dysregulation and over-expression of the candidate TFs. This leads us to speculate that the dysregulation of TFs creates an environment in which several of the survivin expression-correlated genes are highly upregulated.

This leads to the concept of a cancer specific network of survivin expression-correlated genes, which when dysregulated act together to promote CRC development. It is a novel idea that needs to be examined further to see whether such a cancer specific network exists in CRC, compared with normal colonic epithelia. Further, there has been report of "oncogenic addiction"(Jonkers and Berns 2004), (Weinstein 2002) where many oncogenes are needed for the tumor maintenance, at least initially. This gives a window of opportunity where oncogenes might be targeted to suppress or destroy cancer cells. In a similar way, survivin expression-correlated genes should be further examined to determine whether such an "oncogenic addiction" is at play. This will be an interesting area to explore. Such a study is the first step in the direction to understand how dysregulated expression of specific TFs helps in the dysregulation and maintenance of survivin-type cancer gene networks.

This is the very first study where even without any age, race or sex matching it has been shown that the candidate TFs, which are hypothesized to be involved in the regulation of survivin and its expression-correlated genes, are showing over-expression at the mRNA level as well as the protein level, in CRC, compared with the normal colonic epithelium.

Chapter 4

FUTURE WORK

Large-scale network analysis of survivin correlated genes done in our lab has shown that there is a set of genes that are expression-correlated with the survivin gene (BIRC 5). It was predicted that the expression of this set of genes can be co-regulated by a common set of TFs. To identify enriched regulatory elements supporting the hypothesis of co-regulation, the promoter regions of these genes were analyzed to identify shared TREs, which provide information on cognate TFs that may be involved in this co-regulation. Eleven TFs corresponding to those TREs were selected that showed statistically over representation in the set of expression-correlated genes.

As a first step, the differential expression of the candidate TFs was analyzed between tumor tissue and adjacent normal colonic epithelium in mRNA as well as protein level. It was seen that except for *E2F1*, all the remaining TFs showed increased expression in tumor, compared with normal colonic epithelium at the mRNA level. The pattern of over-expression-correlated with the analysis at the protein level where out of the six TFs tested for, (again except for *E2F1*), all five other TFs showed increased expression

The next question is how this project can be taken forward. There may be many directions in which this project can evolve. One path could be to first analyze the TREs in human micro array data sets in normal and malignant colon tissues using PAINT software to find three to four appropriate TREs, which show highest statistically over representation in the set of expression-correlated genes. From this the cognate TFs can be

selected to do chromatin immunoprecipitation (ChIP) assay or electrophoretic mobility shift assay (EMSA). ChIP as well as EMSA is a method of analyzing interactions between protein and DNA/RNA. Hence these will be suitable methods to determine whether the candidate TFs bind to the promoter region of the expression-correlated genes.

The main caveat in doing the ChIP is to find highly specific antibodies that are able to bind with the TFs. Also, several TFs binding to the same promoter region could interfere with immunoprecipitation. These are some issues that have to be addressed before proceeding with the ChIP assay. If specific antibodies are not available then EMSA can be done. With either of these methods the interaction between TFs and the promoter regions of the expression-correlated genes can be uncovered.

Once it has been determined, which candidate TFs are binding to the promoter regions the next step will be to examine the promoter regions of the expression-correlated genes itself. It will be useful to verify the promoter elements of survivin as well as its expression-correlated genes to find whether there are any similar regions that give indication of a better defined set of genes that are co-regulated by the set of TFs. There are many promoter analysis software tools, which can be used to perform promoter analysis like the promoter inspector (Scherf, Klingenhoff et al. 2000). Later, DNA sequencing of the promoters can be done to establish that there are similar promoter elements in survivin as well as its expression-correlated genes.

But after that it becomes imperative to do the analysis using wet lab experiments to show that these promoters are involved in interacting with TFs in co-regulating survivin as well as its expression-correlated genes. Once the approximate putative regions for promoters have been detected, reporter-gene assays based on a series of deletion mutants need to be done to narrow down the DNA regions that play the most

important role in the promoter activities. Once the regulation of candidate TFs *in-vitro* has been demonstrated it is important to test the functionality of the candidate TFs *in vivo.*

Methods based on fluorescence resonance energy transfer (FRET) can be used to detect protein–protein interactions in living cells. Protein–protein interactions can be studied by fusing the proteins of interest to two fluorescent molecules like cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). FRET can be quantified by observing changes in the fluorescence lifetime of the donor using fluorescence lifetime imaging microscopy (FLIM) (Tonaco, Borst et al. 2006). These two powerful techniques can be combined to test the functionality of the candidate TFs in an in-vivo environment like live cells.

Once the efficacy of the candidate TFs has been established in cells, it is important to show it in mouse or in other live models, to regulate the survivin and its expression-correlated genes. This can be achieved using double-knockout mice for survivin or one of its expression-correlated genes and a candidate TF. If over-expression of survivin or its expression-correlated gene can be attained by the introduction of the candidate TF, and later if it leads to tumor formation in double knock-out mice, the regulation of candidate TFs on survivin and its expression-correlated genes is established. The caveat that works against this will be whether the double knock-out survives or succumbs to embryonic or adult lethality.

This study has been done on five patients without age, race or sex matching. It will be worth-while to perform a more controlled study where many of these criteria are taken into consideration. Also it has to be established that the over-expression of the candidate TFs holds true even in a large cohort of patients. Once it has been examined

then the next step will be to determine how to stop the negative regulation, which can lead to CRC.

For this, inhibitors or protein elements can be designed that inhibit the promoters, it will be interesting to find out which are the downstream pathways or proteins that are getting inhibited. It will be important to address the question- Can we design inhibitors or protein elements that can bind to the promoter elements or to the candidate TFs that inhibit survivin-correlated network that are functioning in tumor cells, particularly in CRC?

It has been known that "many of the survivin-binding partners themselves behave as oncoproteins, as they are overexpressed, mutated or functionally exploited in tumors, as opposed to normal tissues" (Altieri 2008) (Supplementary Information S1, Survivin cancer networks). Hence, targeting survivin and its expression-correlated genes using antagonists may be a powerful cancer therapy approach, at least against CRC. Another aspect to keep in mind is that survivin is essential during development, and has important homeostatic functions in certain adult tissue (Li and Brattain 2006) (Fukuda and Pelus 2006). Hence, it becomes imperative to target only those networks that are functional in cancer, leaving untouched the survivin based network that is involved in the maintenance of normal adult tissue homeostasis. How can we design inhibitors that are able to specifically target survivin and its expression-correlated genes in cancer?

Once such antagonists are designed, they can be tested on nude mice xenograft-assay using severely immuno-compromised mice (SCID), or on genetically engineered mice (GEM) (Richmond and Su 2008) where survivin as well as its expression-correlated genes could be mutated or deleted. The particular model system for study should mimic the human CRC as closely as possible. It has to be taken into

consideration that the antagonists against survivin as well as its expression-correlated genes need to be proven safe to use in humans.

Another criterion, which can be put to test, will be, whether it is better to use a single inhibitor (or drug) that targets a lot of the genes or a combination of inhibitors/drugs that target different pathways involved in survivin cancer networks. Also, the same drug in humans can act differently compared with an immunologically compromised mouse model. The next aspect to take into consideration will be whether these inhibitors are safe to use in humans within a clinical perspective.

All these questions have to be answered before the drug or drugs are taken to pre-clinical or clinical trials. The design or discovery of survivin cancer network antagonist or antagonists with fewer side effects will be a major step in the diagnosis and treatment of CRC.

4.1 Perspectives

Recently there has been a technological leap in the arena of bioinformatics based on statistical, mathematical, and data mining tools. There also have been technological advances in the area of gene expression studies using microarray platforms, in which thousands of genes can be analyzed in a single experiment. Until now, researchers have been able to analyze only a handful of genes at a time. The era of bioinformatics has opened numerous opportunities for scientists and researchers. Microarray profiling of genes helps in analyzing expression patterns of thousands of genes active in a cell at a given time, while bioinformatics tools help to analyze and interpret the biological data. This strategy helps in developing gene expression based diagnostic tests that can be tailored to individual patients.

In the case of changes in survivin and its expression-correlated genes,the development of a diagnostic test or a survivin cancer network antagonist based on the

expression pattern and transcriptional regulatory network will be a major milestone in the treatment of CRC.

High-throughput studies that help in the identification of "red-dot genes", which can be targeted specifically to stop the progression of cancer, are invaluable tools in cancer therapy. Red dot genes are "those genes whose mutation occurs early in oncogenesis and dysregulates a key pathway that drives tumor growth in all of the subclones" (Simon 2005). Survivin and its expression-correlated genes may be considered red dot genes. A "single gene at a time" approach may not be useful in cancer treatment, as numerous signaling pathways are dysregulated and many times proteins function as moon-lighting proteins having regulatory functions in multiple pathways. Thus a concerted effort to target multiple pathways at the same time is needed. Survivin and its expression-correlated genes can be considered a study in that direction with a CRC perspective. The study of survivin and its expression-correlated genes can be considered an invaluable effort that would shed light into many pathways that are involved in CRC, which can in turn lead to the development of powerful drugs that help to conquer CRC.

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