The Pennsylvania State University
The Graduate School
College of Medicine

POTENTIAL OF PROSTATE APOPTOSIS RESPONSE PROTEIN-4 (PAR-4) IN
COLON CANCER THERAPY

A Dissertation in
Integrative Biosciences
by
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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2011
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Abstract

Prostate apoptosis response protein 4 (Par-4) has been shown to induce apoptosis in cancer cells. However, little has been published on the role of Par-4 in colon cancer. The purpose of this dissertation is to demonstrate that Par-4 has potential in colon cancer therapy by virtue of its impact on colon cancer cell apoptosis. To achieve this goal, Par-4 was overexpressed in the colon cancer cell line HT29. Par-4-overexpressing cells were more susceptible to apoptosis induced by the colon cancer chemotherapeutic agent 5-fluorouracil (5-FU). Not only does Par-4 have potential in vitro, it also inhibits colon cancer growth in vivo. Par-4 overexpressing tumors grew more slowly and to a smaller mass than wild type HT29 tumors. Caspase-9 cleavage was increased in the Par-4 tumors. In addition to demonstrating the pro-apoptotic effects of overexpressing Par-4 in colon cancer, the potential of activating endogenous Par-4 was explored. In colon cancer, the protein levels and the kinase activity of the nonreceptor tyrosine kinase, c-Src, increase with tumor progression. One of the downstream effectors of c-Src is Akt1. Akt1 has been shown to inhibit the pro-apoptotic activity of Par-4 in prostate cancer cells. Therefore, the possibility of activating Par-4 by inhibiting c-Src was investigated. Colon carcinoma cell lines were treated with the Src kinase inhibitor (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine (PP2) in combination with 5-FU. Treating cells with PP2 and 5-FU resulted in reduced interaction of Par-4 with Akt1 and with the scaffolding protein 14-3-3σ, and mobilization of Par-4 to the nucleus. Par-4 was shown to interact not only with Akt1 and 14-3-3σ, but also with c-Src. Overexpression of c-Src induced the phosphorylation of Par-4 at tyrosine site/s.
Thus, endogenous Par-4 can be activated by inhibiting Src kinase with a pharmacological inhibitor and adding a chemotherapeutic agent. The activation of the pro-apoptotic protein Par-4 is a novel mechanism by which apoptosis occurs with a Src kinase inhibitor and 5-FU. In this dissertation, therefore, I demonstrate that a) Par-4 overexpression in combination with the apoptotic and chemotherapeutic agent 5-FU causes cell death in colon cancer cells; and b) endogenous Par-4 can be activated in colon cancer cells by Src inhibition and 5-FU treatment. Thus, the first steps toward attainment of the long-term goal of elucidation of the Par-4 function in colon cancer have been made. With the understanding that Par-4 can be activated in colon cancer, and that Par-4 can promote apoptosis in colon cancer and decrease colon cancer growth, strategies can be designed to exploit the pro-apoptotic activity of Par-4 in colon cancer therapy.
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### Abbreviations

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<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis Inducing Factor</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine mononucleotide phosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>caspase-activated DNase</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>cIAP</td>
<td>Cellular Inhibitor of apoptosis</td>
</tr>
<tr>
<td>c-FLIP</td>
<td>Caspase-8 homologous FLICE Inhibitory Protein</td>
</tr>
<tr>
<td>DAP</td>
<td>Death-Associated Protein</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DISC</td>
<td>Death Inducing Signaling Complex</td>
</tr>
<tr>
<td>Dlk</td>
<td>DAP-like kinase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dnmt</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>DR5</td>
<td>Death receptor 5</td>
</tr>
<tr>
<td>dUTP</td>
<td>Deoxyuridine triphosphate</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-Associating protein with Death Domain</td>
</tr>
<tr>
<td>FKHRL1</td>
<td>Forkhead (Drosophila) homolog (rhabdomyosarcoma) like 1</td>
</tr>
<tr>
<td>FLICE</td>
<td>FADD-Like Interleukin-1-β-Converting Enzyme</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of Apoptosis Protein</td>
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<tr>
<td>IKK</td>
<td>IkB Kinase</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear Export Sequence</td>
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<tr>
<td>NFκB</td>
<td>Nuclear Factor Kappa-light chain enhancer of activated B cells</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Sequence</td>
</tr>
<tr>
<td>Par-4</td>
<td>Prostate apoptosis response protein-4</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP-Ribose) Polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDK</td>
<td>Phosphoinositide-Dependent Kinase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PP2</td>
<td>4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SAC</td>
<td>Selective for Apoptosis induction in Cancer cells</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Smac</td>
<td>Second mitochondria-derived activator of caspase</td>
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<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>Top1</td>
<td>DNA Topoisomerase I</td>
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<tr>
<td>TRAF</td>
<td>TNF Receptor Associated Factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-Related Apoptosis Inducing Ligand</td>
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<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
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<tr>
<td>XIAP</td>
<td>X-chromosome-linked Inhibitor of Apoptosis Protein</td>
</tr>
<tr>
<td>ZIP</td>
<td>Zipper-interacting protein kinase</td>
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Acknowledgments

The processes described in this dissertation do not involve only one protein. In fact, Par-4 has numerous interactors that work with it to accomplish its function. Similarly, this research was not made possible by the efforts of one person alone. Although they are not given authorship in this dissertation, it is with them that I have accomplished the work reported here.

First, I would like to acknowledge my dissertation adviser, Dr. Rosalyn B. Irby. Without her support, I would not have even been able to leave my fruitful position as her research assistant and continue my pursuit of a Ph. D. I still remember the day when I asked her for the chance to work with Par-4. With her approval of my request, my journey with Par-4 progressed. Her guidance has been invaluable. Very close to the beginning of this project, Dr. Irby has trusted my ideas and has allowed me to pursue them. I am truly grateful for the independence that she has given me. Dr. Irby has encouraged me to present my work in research meetings and publish my research findings. The work presented here is only part of the research endeavors of the Irby laboratory. However, Dr. Irby has considered me as an able research colleague by allowing me to work with her and her collaborators. This has given me authorship in papers that are not even covered in this dissertation. However, through these research collaborations, I have gained more insight on the potential of Par-4 in colon cancer therapy.

The research work presented here was financially supported by two grants. One is a grant to Dr. Irby from the Barsumian trust. The other is a grant from the American Federation for Aging Research. The funding that they entrusted to us demonstrates that we all can contribute to the treatment of disease in ways that we can.

This dissertation work was conducted under the expert supervision of my committee members. They did not mince words in pointing out to me that my Western blot results needed to be better, experiments needed to be repeated or my figures needed to be improved. Although to a few, these comments may bring frustration. However, to me, these comments pushed me to do my best in all aspects of research -- the design of the experiments, the performance of my experiments, and the presentation of my research findings.
I am indebted to Penn State University for their trust in me. Choosing me based on application materials and a phone interview was a great risk. When I asked to return to resume my Ph D studies, they chose to trust me again. I hope that I had shown through my work and through my conduct that their trust in me proved beneficial to them and to society.

The Penn State College of Medicine is truly a great place to work. This research progressed because my colleagues in the college have shared with me their time and resources. I would like to acknowledge the members of the Loughran lab of the Penn State Cancer Institute. Because of their graciousness, I have been able to use equipment that the Irby lab did not have. Dr. Nicole Keasey of the Claxton lab has been patient with me even when I sometimes used the cell culture hood for hours. Because of the work of my colleagues in the different Core Facilities of the college, I have been able to perform many of my experiments. The Supply Center staff have been faithful in assisting me in my purchase of needed supplies. Wade Edris of the Imaging Core Facility, Nate Sheaffer and Dr. Dave Stanford of the Flow Cytometry Core and Dr. Kang Li of the Histology Core helped me in the performance of my experiments by providing their expertise. The staff of the Central Animal Quarters have been helpful and patient as I conducted the mouse studies reported here.

I would also like to acknowledge my parents whose choices and examples have been used by God to prepare and equip me for this endeavor. I will not be able to thank them enough.

Many of the research figures in the Introduction section of this dissertation are the artwork of Walt Kline II. However, his contribution to my work has not only been his creative renderings. It is because he has been willing to let me work weird and long hours, not have to do my chores for awhile, read and work in the laptop in the car while traveling, that this research continued. Each experiment was conducted with his prayer support and listening ear. I know that it would have been hard for an architectural designer to listen about proteins and mice and keep interest. However, he did. I am grateful. He is one of two to whom I want to dedicate this dissertation to. Through Walt, I have the chance of touching and being touched by three children – Brendon, Genoah and Kailey. They also made sacrifices in their young lives so that the research can be done.

The second and yet, the more important One to whom I would like to truly dedicate this research is Jesus Christ. I would not even be here writing this dissertation were it not for Him. He is the One who has enabled me to live, and thrive through the different challenges that faced me before and through this dissertation work. It is through Him, for Him, and in Him that I live.
Chapter 1

Introduction

1.1 Colon Cancer

Colon cancer is cancer that forms in the large intestine or colon. Cancer that forms in the last several inches of the colon is called rectal cancer. Thus, the term colorectal cancer is used to refer to both colon and rectal cancers. Colorectal cancer is the third most commonly diagnosed malignancy in both men and women. It is the third leading cause of cancer-related death in the US (1). Although epidemiological studies usually consider colon and rectal cancers together, there are differences in these two cancers. One important difference lies in the management of the disease. The benefit of radiation therapy in combination with chemotherapy has been shown for rectal cancer (2, 3). In colon cancer, however, radiation has been suggested only for certain subsets of patients, namely, those who are at risk of local recurrence (4, 5). Given the distinction between colon and rectal cancer, this dissertation work is focused on colon cancer.

Survival rates with colon cancer depend largely on the pathologic stage of the disease. The most advanced stages involve the spreading of the colon cancer cells to distant organs, such as lung and liver. This spread is called metastasis. Cancers are staged based on the size of the tumor or the extent that it has penetrated the different layers of the colon, on the number and location of the lymph nodes involved, and the incidence of
distant metastasis (6) (Figure 1-1). When the colon cancer cells are only found in the innermost lining (mucosa) of the colon, the cancer is stage 0 or carcinoma in situ. Once the cancer cells spread to the submucosa or the muscle layers, the cancer is adjudged as Stage I. Stage II colon cancer is cancer that has spread through the muscle layers of the colon and even has passed this layer, but has not involved the lymph nodes. Once it has been found that the cancer has also spread to the lymph nodes, the colon cancer is staged as Stage III. In Stage IV cancers, the cancer has spread to the other organs (Figure 1-1). The treatment for Stage 0, I, and II colon cancers is surgical removal of the tumor. The 5-year survival rate for Stages 0 to II is 90.1%. On the other hand, the 5-year survival rate drops to 69.2% for Stage III cancers. It is hypothesized that the reason behind the lower survival rate of Stage III cancer patients than that of earlier stages is due to the presence of residual cancer cells that were not removed by surgery (7). To increase treatment success for stage III cancers, chemotherapy is given after surgery (referred to as adjuvant chemotherapy). The purpose of adjuvant chemotherapy is to kill residual disease that could have been undetected during the time of surgery. Clinical studies have shown that adjuvant chemotherapy is superior to surgery alone in preventing disease recurrence (8) and increasing disease-free survival and overall survival (9). For Stage IV cancer, the 5-year survival rate is 11.7% (10). The treatment strategy for Stage IV disease largely depends on the location of the cancer cells. The predominant site of colon cancer metastases is the liver, in part because the liver is the first major organ reached by venous blood draining from the colon, (11, 12). Surgery, if possible, is performed to remove the tumors. Only 10-20 % of patients, however, are good candidates for surgery (13). Surgery is not usually considered for
patients who have extrahepatic disease (14-18) or diffuse hepatic disease (19). Surgery is also contraindicated when the metastases are too large, ill-located, or multinodular (13, 18). Stage IV patients can be treated with chemotherapy at the onset (referred to as first-line therapy) (20, 21). Despite long-standing efforts to improve treatment, however, the current median overall survival for metastatic colorectal patients treated with chemotherapy is about 2 years (22). Chemotherapy can be performed either before or after surgery. Adjuvant chemotherapy (chemotherapy after surgery) has not been conclusively shown to be beneficial for Stage IV patients. Although there has been a trend towards an increase in survival in patients treated with chemotherapy after surgery versus patients that underwent surgery alone, the differences in survival rates are not statistically significant between the two patient groups (23). While chemotherapy after surgery has yet to be proven advantageous, using chemotherapy before surgery has been proven to be beneficial. Chemotherapy has resulted in reduction in tumor size and decrease in tumor markers (carcinoembryonic antigen and carbohydrate antigen 19-9) in patients previously adjudged as poor candidates for surgery (18, 24, 25). Possibly because of the decrease in size and number of the liver metastases as a result in part of chemotherapy, surgical removal of the metastases has become feasible in these patients. Given the role of chemotherapy in treatment of colon cancer, in the next section, the current chemotherapeutics for colon cancer are discussed.
Figure 1-1. Different stages of colon cancer. The progression of a colon tumor from Stage 0 to Stage IV. In Stage 0 colon cancer (the smallest tumor mass in the leftmost part of the colon in the figure), the tumor cells are localized in the inner lining of the colon. As the disease progresses, however, tumor cells invade and pass through the other layers of the colon, and eventually move to the lymph nodes and spread to organs. (Image used with permission from Ms. Terese Winslow/ National Cancer Institute).
1.2 Current Chemotherapeutic Strategies against Colon Cancer

1.2.1 5-Fluorouracil

The mainstay of colon cancer therapy is 5-fluorouracil (5-FU). The adaptation of 5-FU as a cancer chemotherapeutic came, in part, from the observation that rat liver tumor cells use uracil for nucleic acid formation at a significantly higher degree than their normal liver cell counterparts (26). When 5-FU was first reported to be synthesized, it was also tested for its ability to inhibit tumor growth. In rats and mice treated with 5-FU, the growth of tumors was decreased (27). Efforts were initiated to treat human cancers, including colon cancer. 5-FU was first given as a single intravenous injection for 5 days. However, tumor regression was only observed when toxic 5-FU concentrations were used. Some of the toxic effects included nausea, vomiting, diarrhea, and drops in white blood cell and platelet count (28). Thus, for decades, numerous studies have been done to optimize the dose (29), schedule (30-32) and mode of administration (33, 34) to maximize therapeutic effect and minimize toxicity. Currently, 5-FU is more commonly given to patients, at least in the US, in regimens that consist of combinations of bolus injection and continuous intravenous infusions.

What has been considered as the primary mode of action of 5-FU is inhibition of the enzyme thymidylate synthase. In cells, 5-FU can be converted to 5-fluoro-2’-deoxyuridine-5’-monophosphate (FdUMP) (35, 36) (Figure 1-2). FdUMP forms a
complex with thymidylate synthase, in the presence of the cofactor 5,10-methylenetetrahydrofolate (5,10-CH2-THF) (37-39). Thymidylate synthase is the enzyme that catalyzes the transfer of a methyl group from 5,10-CH2-THF (40) to deoxyuridine monophosphate (dUMP), forming deoxythymidine monophosphate (dTMP) (41). The dTMP is converted to deoxythymidine triphosphate, one of the building blocks of DNA. Because the carbon-fluorine bond of FdUMP is stronger than the corresponding carbon-hydrogen bond in dUMP, the complex of FdUMP with thymidylate synthase is very stable (38). Thus, less amount of enzyme is available to convert dUMP to dTMP. Consequently, deoxythymidine nucleotide levels decrease (42, 43). This results in the inhibition of DNA synthesis (44). Another consequence of thymidylate synthase inhibition is dUMP and dUTP accumulation (42, 43, 45, 46). The accumulated dUTP can be misincorporated into DNA by DNA polymerase. The DNA repair pathway can excise the misincorporated uracil (47, 48). It has been proposed that because DNA repair requires DNA synthesis, the excised dUTP is reincorporated and the process keeps on repeating in what is referred to as futile cycling. DNA strand breakage ensues (49) as the repair mechanism fails.

5-FU affects both DNA and RNA. 5-FU can be converted to 5-fluorouridine triphosphate (FUTP) (Figure 1-2) and be incorporated into RNA (36, 50, 51) by RNA polymerase. The inclusion of 5-FU into RNA has been correlated with 5-FU cytotoxicity (52). 5-FU can be incorporated into the 45S and 32S preribosomal RNA, inhibiting the processing of these molecules into the 28S and 18S RNA components of the ribosome (53). In
Figure 1-2. Anabolism and effects of 5-FU. 5-FU can be metabolized in cells via different pathways. Interconversion can occur and thus, 5-FU treatment can have more than one effect. The fluorine residue is shaded in gray (image courtesy of Walter J. Kline II).
addition, 5-FU can also be inserted into the U2 spliceosomal snRNA. This results in inhibition of pre-mRNA splicing (54). Thus, 5-FU can inhibit key processes involving RNA, namely pre-mRNA splicing, ribosome formation, and protein synthesis.

Much research has been done to increase response to 5-FU. One strategy that has been explored is to increase the duration of the 5-FU-mediated inhibition of thymidylate synthase. As has been mentioned above, 5-FU forms a complex with the enzyme in the presence of the cofactor 5,10-CH2-THF. The levels of this cofactor, however, are limited physiologically by the conversion of 5,10-CH2-THF to dihydrofolate and 5-methyltetrahydrofolate. Although this steady-state expression of 5-10-CH2-THF is sufficient for the normal growth of cells, it does not promote maximal binding of FdUMP to thymidylate synthase (55). In addition, increasing the levels of the cofactor is advantageous because it decreases the dissociation of the FdUMP-TS complex (56). Thus, to favor the formation and increase the stability of the FdUMP-TS complex, efforts to increase the levels of 5-10-CH2-THF have been made. Cells have been treated with folinic acid or leucovorin, the formyl derivative of the cofactor. Folinic acid is converted into 5-10-CH2-THF in cells. Incubation of cells with folinic acid and folate have increased response to 5-FU and to FdUMP (55, 57). This is due at least in part to the stabilization of the FdUMP-TS complex and therefore, prolongation of the inhibition of thymidylate synthase (58). Given the favorable *in vitro* effects of folinic acid in combination with 5-FU, clinical studies have been conducted to adopt the combination. Administering the combination has increased overall survival and time to disease
progression versus giving patients 5-FU alone (59, 60). The response rate to the 5-FU/leucovorin combination has been increased to 30% (32), from a 20% response rate with 5-FU monotherapy. Other compounds have been added to 5-FU and leucovorin to increase treatment success.

1.2.2 Oxaliplatin

Oxaliplatin, a platinum (II) complex of trans-l-diaminocyclohexane, was first synthesized (61) because of the effectiveness (62) and toxicity (63) of previously synthesized platinum compounds like cis-dichlorodiamine platinum II (cisplatin). Oxaliplatin has been found to be as effective as and less toxic than cisplatin (61). Cisplatin has been shown to increase the cytotoxicity of 5-FU. Cisplatin induces an increase in 5,10-CH2-THF pools and in FdUMP - thymidylate synthase complex (64). Because of the toxicity of cisplatin, however, the possibility of combining oxaliplatin with 5-FU was explored. The 5-FU-leucovorin (LV)-oxaliplatin treatment resulted in 50-60% response rate (20, 65). Given the favorable response to the triple combination, subsequent studies that were performed sought to optimize dose and schedule of administration of the three drugs (66, 67). The term FOLFOX has been used to refer to the combination of the chemotherapeutics (68).
Oxaliplatin has been shown to bind to DNA, similar to cisplatin. Oxaliplatin forms adducts between two adjacent guanine bases in a single DNA strand (69, 70). The structure of the oxaliplatin-DNA adducts are dissimilar, however, with the cisplatin-DNA adducts by virtue of the diaminocyclohexane (DACH) ring of oxaliplatin (Figure 1-3). The cisplatin adducts are recognized by the mismatch repair proteins (71, 72), resulting in the eventual engagement of apoptosis. Cells, especially cancer cells, that have a defective mismatch repair system, escape this killing mechanism and exhibit resistance to cisplatin (73). On the other hand, the hydrophobicity and bulkiness of the DACH ring of oxaliplatin may prevent the binding of the mismatch repair proteins (74) This can partly explain the observation that cells that have lost a functional mismatch repair system lose their susceptibility to cisplatin but not to oxaliplatin (73).

Figure 1-3. Chemical Structures of cisplatin and oxaliplatin. The diaminocyclohexane ring imparts a bulk and hydrophobicity to oxaliplatin that contributes to its resistance to mismatch repair-mediated removal from the DNA.
The adducts formed by oxaliplatin and cisplatin are bypassed during DNA synthesis by DNA polymerase β. The polymerase predominantly catalyzes misinsertions opposite the adducts (75). Thus, mutagenesis occurs.

1.2.3 Irinotecan

In the search for novel antitumor agents, a plant alkaloid, camptothecin, was isolated and shown to be effective against mouse leukemic cells and rat tumors (76). To increase antitumor activity and lessen toxicity, derivatives of camptothecin have been synthesized and tested. The water-soluble derivative, CPT-11 or irinotecan is effective against different mouse tumors (77) and human tumor xenografts in nude mice (78). It has an additive effect in combination with 5-FU treatment in vitro (79, 80). In vivo, the bulky piperidino side chain of irinotecan (77) is removed by carboxylesterase, yielding SN-38 (81) (Figure 1-4). Irinotecan has been combined with 5-FU and leucovorin in the clinic, resulting in increased response rate, time to progression, and overall survival in comparison to treatment with the 5-FU/leucovorin combination (82).

Irinotecan inhibits the enzyme DNA topoisomerase I. Thus, in contrast to 5-FU and leucovorin, irinotecan action does not involve inhibition of thymidylate synthase (80). The inhibition of DNA topoisomerase I (Top1) by irinotecan is particularly important in colon cancer therapy. It has been shown that Top1 expression is 14-16-fold higher in colon cancer tissue in comparison to normal colon mucosa (83). DNA topoisomerase I
Figure 1-4. Chemical structures of camptothecin, irinotecan and SN-38 (Image courtesy of Walter J. Kline II)

catalyzes unwinding of the DNA in an ATP-independent manner. The unwinding of DNA is critical for DNA replication (84) and transcription (85, 86). Top1 cleaves one of the DNA strands, enables the smooth rotation of the complementary DNA around the first strand, and reseals the nick in the first strand (87, 88).

Research on how camptothecin and its derivatives, e.g. irinotecan and SN-38, affect Top1 function have focused mostly on camptothecin. Thus, these studies are described herein. Camptothecin binds reversibly (89) to the DNA-Top1 complex consisting of Top1 covalently linked to the 3’-DNA termini of the nicked DNA (referred to as cleavable complex). The binding of camptothecin (CPT) to the cleavable complex inhibits the Top1-catalyzed religation of the nicked DNA (90). Moreover, the CPT-induced stabilization of the Top1 on the DNA can possibly inhibit the movement of RNA and
DNA polymerase along the DNA strand, and stall RNA and DNA synthesis (91). It has been proposed that the CPT-DNA-Top1 complex collides with the DNA replication fork, resulting in a double-strand DNA break at the fork (92). A similar model has been suggested for transcription, where the CPT-DNA-Top1 complex collides with the elongating RNA polymerase. This also results in strand breaks (93) (Figure 1-5). Although most of the studies on the effects on Top1 have been on camptothecin, there is evidence that similar mechanisms occur with the irinotecan metabolite SN-38. SN-38, like camptothecin, induces DNA single strand breaks. The complex formed by SN-38 with DNA-Top1 has been shown to be more stable than the complex formed by CPT (94, 95).

Currently, irinotecan is administered in combination with 5-FU and leucovorin. In the IFL or Saltz regimen, the three drugs are administered concurrently (96). In the FOLFIRI regimen, the irinotecan is administered concurrently with leucovorin. This is followed by a bolus injection and a continuous infusion of 5-FU (67). Results of in vitro experiments have shed light on the mechanism behind the therapeutic benefit of combining irinotecan with 5-FU/LV, especially in a sequential manner. Pretreatment of human intestinal adenocarcinoma cells with the active metabolite of irinotecan, SN-38, results in an increase in dTTP and decrease in dUTP levels. The increase in dTTP is expected to inhibit dCTP deaminase and thymidine kinase (42). Consequently, dUMP pools are decreased. With the decrease in dUMP, there is less competition with FdUMP for the active site in thymidylate synthase. Therefore, the effect of 5-FU on thymidylate
synthase is enhanced with SN-38 pretreatment (97). Irinotecan (CPT-11) itself has been shown to be effective, whether added before or simultaneously with 5-FU, in prolonging thymidylate synthase inhibition. The synergy of these two drugs also affects

**Figure 1-5. Mechanism of inhibition of DNA topoisomerase I (Top1) by irinotecan**

I. DNA Topoisomerase I (Top1) interacts with the DNA helix; II. DNA cleaves one DNA strand. III Irinotecan (CPT-11) keeps DNA from religating the nicked strand and forms a complex with Top1 and the DNA. DNA polymerase is replicating the DNA in the direction of the arrow. Once DNA pol reaches the CPT-DNA-Top1 complex, (where the DNA strand has not been religated), a double strand break ensues. Presumably, the lagging strand is also replicated. The double stranded DNA (light and dark blue strands) formed using the lagging strand has a single strand break (image courtesy of Walter J. Kline II).

cells at the DNA level. Increases in protein-DNA complexes (the cleavable complexes) and in 5-FU nucleotide incorporation into DNA were observed concomitantly. This indicates that cumulative DNA damage occurs with the irinotecan (CPT-11) and 5-FU
combination as CPT-11-DNA-Top1 complexes are formed and 5-FU nucleotides are incorporated into DNA (80).

The chemotherapeutics that have been discussed are the main components of colon cancer chemotherapy regimens that are currently being used in the US. However, because these agents target nucleotide metabolism, particularly DNA synthesis, they affect normal and cancer cells. Thus, side effects are prevalent. To address this, more targeted agents are being explored for use. Many of these include monoclonal antibodies against receptor tyrosine kinases. At this juncture the direction of colon cancer chemotherapy research is towards the development of agents that are selective for cancer cells.

1.3 Targeting Apoptosis in Colorectal Cancer Therapy

1.3.1 Roles of Apoptosis in Normal Function and Disease

More than 80% of patients experience side effects from chemotherapy (98). Side effects from chemotherapy are results, at least in part, of the effects of drugs on normal cells. From the information presented in the previous section, current chemotherapeutic regimens target pathways that are common to normal and cancer cells; namely, DNA and RNA metabolism. A strategy, however, to avoid side effects is to target a
characteristic that is exhibited by cancer cells and not (or at least, to a lesser degree) by normal cells. One of the hallmarks of cancer cells is resistance to programmed cell death or apoptosis. One observation that may indicate that apoptosis resistance is relevant in the context of colon cancer is the detection of low apoptotic indices in colon cancer tissues of patients that had poor survival rates (99).

Apoptosis or what may be referred to as programmed cell death, is a physiological process to induce specific cells to die. A specific set of morphological and biochemical changes has been associated with cells undergoing apoptosis. A characteristic morphological feature of apoptosis is the coalescing of condensed cytoplasm and nuclear chromatin (called apoptotic bodies) (100). These can be observed under the microscope after nuclear staining. The characteristic biochemical effects of apoptosis include mitochondrial membrane depolarization (101), activation of cysteine proteases called caspases (102), internucleosomal DNA degradation (103), and externalization of the lipid phosphatidylserine at the plasma membrane (104). Given the advancements in assay techniques, it has been proposed that apoptosis be defined more by biochemical changes, rather than morphological alterations. Morphologic observations can be skewed by analyst bias. In addition, biochemical assays allow quantitation and thus, comparisons between different treatments or conditions. However, in certain situations of apoptosis, not all the biochemical changes that have been mentioned are detectable. A particular biochemical effect can also have other causes aside from apoptosis. Thus,
it has been advised that more than one biochemical parameter be assessed before confirming or discounting apoptosis (105).

The term programmed cell death was first used to refer to the loss of specific muscle cells in the moth larva at a defined point in moth development (106). The death of these cells occurred only after the emergence of the adult moth. This indicates that this particular mode of cell death was not a random event but the result of a systematic process. Evidence that cell death is a genetically defined process has been initially gathered from studies on the nematode *Caenorhabditis elegans*. This organism has 1090 somatic cells, 131 of which die during specific stages of development. Mutations in the *ced* genes blocked cell death in *C. elegans* (107, 108). This indicated that programmed cell death is driven by expression of certain genes. Eventually, apoptosis has been defined more strictly as a type of programmed cell death where a specific set of morphological and biochemical changes are involved. Other types of programmed cell death have been characterized, like paraptosis (109) and necroptosis (110), that are also dependent on expression of specific genes (111). However, their morphological and biochemical consequences are distinct from that of apoptosis. The focus of this dissertation is on the apoptotic process; other modes of programmed cell death are not discussed.
In mammalian development, particular cells need to die to form the body properly, especially fingers and toes, and to shape the organs. The importance of apoptosis in development can be gleaned from the phenotypes of mice where genes involved in apoptosis have been knocked out. In mice where the mammalian homologue of *ced-3* has been knocked out, abnormal brain development is observed. Parts of the brain protrude out in association with skull defects. Portions of the neuroepithelium protrude into the retinal area, causing compression of the lens. These morphologies have been attributed to decreased apoptosis in the embryonic brain of the knockout mice (112). In another set of studies, mice that had a null allele of the mammalian homologue of *ced-4*, have been developed and studied. These mice had craniofacial alterations, lens and retinal abnormalities, and interdigital webs, in addition to brain abnormalities. This indicates that apoptosis is necessary for the proper development of the skull, face, eyes, and limbs (113).

Clonal deletion is another example of a physiological function where apoptosis is critical. Clonal deletion or negative selection is the process by which immature T lymphocytes that recognize self-antigens are destroyed in the thymus (114). Failure to eliminate these self-reactive T cells can result in autoimmune reactions. These self-reactive T-cells are induced to die by apoptosis (115, 116). Apoptosis is initiated when the thymocytes’ T-cell-receptors interact with antigenic peptides in antigen-presenting cells in the thymus. In the thymus, only cells that are expressing self-antigens are
expected to be present. Thus, if an immature T-cell interacts with a peptide, then that particular T-cell has specificity for a self-antigen, and must be eliminated.

Although apoptosis is necessary for normal function, it is also involved in certain pathological states. In autoimmune diseases, namely, rheumatoid arthritis, systemic lupus erythematosus, type I diabetes, and multiple sclerosis, the expression of genes involved in apoptosis has been observed (117). Autoimmune diseases involve the undesired attack of immune cells on different tissues. In type I diabetes, for instance, self-reactive T cells destroy the pancreatic β cells (118). An anti-apoptotic protein, caspase 8-homologous FLICE (Fas-associated death-domain-like interleukin 1β-converting enzyme)-inhibitory protein (known as c-FLIP), has been found to be upregulated in self-reactive thymocytes in a mouse model of type I diabetes (119). The inhibition of apoptosis of these thymocytes has enabled them to survive, instead of being eliminated by clonal deletion or negative selection. Subsequently, they leave the thymus and attack the pancreas, causing type I diabetes.

Autoimmune diseases are only one subset of pathologies where dysregulation of apoptosis is involved. In cancer, apoptosis is important in tumor initiation, progression, and treatment resistance. In follicular lymphoma, the overexpression of an anti-apoptotic protein Bcl-2, along with the activation of an oncogene, is responsible for the etiology of the disease (120). Another anti-apoptotic protein, Bcl-xL, is involved in
multiple drug resistance. Lymphoid cells that express Bcl-xL demonstrate resistance against six different chemotherapeutic agents and against gamma-irradiation (121). Cancer cells possess mechanisms that enable them to escape killing by the apoptotic mechanisms. An understanding of the different pathways of apoptosis is critical to therapeutic success in cancer, in other diseases where dysregulation of apoptosis occurs. These pathways of apoptosis are the subject of the next section.

1.3.2 Apoptosis pathways

Cells are induced to die in response to certain extracellular or intracellular signals. When apoptosis results from extracellular stressors, it is termed as extrinsic apoptosis. The extrinsic apoptotic pathway is initiated by the interaction of extracellular protein ligands, the death ligands, with membrane death receptors (Table 1-1). The death ligands that have been identified belong to the tumor necrosis factor (TNF) superfamily of proteins. Death ligands are expressed by cells as homotrimeric (122, 123) type II transmembrane proteins (124) (Figure 1-6). Type II transmembrane proteins are
Table 1-1. Death Ligands and the Death Receptors that they interact with

<table>
<thead>
<tr>
<th>Death Ligands</th>
<th>Death Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor necrosis factor α</td>
<td>TNF Receptor 1 (TNFR1)/p55/p60</td>
</tr>
<tr>
<td>Lymphotoxin α₃</td>
<td>TNF Receptor 1 (TNFR1)/p55/p60</td>
</tr>
<tr>
<td>Lymphotoxin α₂β₁</td>
<td>TNF Receptor 1 (TNFR1)/p55/p60</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas/CD95/Apo-1</td>
</tr>
<tr>
<td>TNF-related apoptosis inducing ligand (TRAIL)/Apо2 Ligand</td>
<td>TRAIL receptor-1/Death receptor 4, TRAIL receptor-2/Death receptor 5</td>
</tr>
<tr>
<td>Apo3 Ligand</td>
<td>Apo-3/TRAMP/ Death receptor 3</td>
</tr>
<tr>
<td>Unidentified (as of yet)</td>
<td>Death receptor 6</td>
</tr>
</tbody>
</table>

References: (125-128)

proteins that traverse the cell membrane once, with their amino-termini inside the cell and their carboxy termini exposed to the extracellular space. The C-terminal domain is conserved among the different death ligands and is referred to as the TNF homology domain (THD). The THD is the region involved in interactions with the death receptor. The trimeric THD interacts with the cysteine-rich domains of three death receptor chains. In the past, it was hypothesized that death ligand binding induces association of the death receptors into trimers (129). However, recent evidence suggests that death receptor molecules can oligomerize, even in the absence of a death ligand, via their pre-ligand-binding assembly domains (PLAD). This oligomerization is necessary for the binding of death ligand (130). Consequent to death ligand-receptor binding, a
Figure 1-6. **Extrinsic activation pathway of apoptosis.** The interaction of the trimeric death ligand with the death receptor activates the extrinsic pathway of apoptosis. FADD or TRADD are recruited to the death receptor via their death domain (DD). Consequently, procaspase-8 (or procaspase-10)[not shown in the figure] interacts with FADD/TRADD through interactions between their death effector domains (DED). The complex formed by the death receptor, FADD/TRADD, and procaspase-8 (or -10) is called the death-induced signaling complex (DISC). Procaspase-8 is subsequently activated. Active caspase-8 cleaves caspase-3. The activation of caspase-3 results in apoptosis (image courtesy of Walter J. Kline II).
conformational change in the receptor occurs. This exposes protein structures in the intracellular region of the receptor called death domains (131).

Cytoplasmic adaptor proteins [i.e. Fas-associated protein with death domain (FADD); TNFR-associated protein with death domain (TRADD)] that contain death domains associate with the exposed death domains of the receptor. Each type of death receptor recruits a specific adaptor protein (Table 1-2) (132-134). The death domain-containing adaptor proteins can also have regions called death effector domains (DED). It is via these death effector domains that the adaptor proteins recruit the procaspases-8 (135-137) and -10 (138-140) to the receptor. The receptors, adaptor proteins, and procaspases form what is referred to as the death-inducing signaling complex (DISC) (141). The concentration of the procaspases in the DISC activates the procaspases (137, 138) by a yet unestablished mechanism. A number of hypotheses have been proposed to explain how the procaspases are activated at the DISC. In the induced proximity model, it is hypothesized that the clustering of the procaspases promotes their self-processing (142). The proximity-induced dimerization model specifies that it is not the nonspecific clustering that is critical for activation. Instead, the recruitment of the procaspases at the DISC enables the dimerization of the procaspases, resulting in procaspase activation (143). The third hypothesis, the induced-conformation model, proposes that the interaction of the procaspase with the adaptor protein results in a conformational change in the active site of the procaspase. This activates the procaspase (144). Activated procaspases-8 and -10 cleave the effector caspase
procaspase-3. Caspase-8 can also cleave the effector caspase procaspase-7 (145).

Given that the consequences of the activation of procaspases-3 and -7 are common regardless of whether external or internal stimuli initiated apoptosis, these effects will be discussed later in the discussion.

Table 1-2. Death Receptors and the Adapter Proteins that they interact with

<table>
<thead>
<tr>
<th>Death Receptors</th>
<th>Adaptor protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF Receptor 1 (TNFR1)/p55/p60</td>
<td>TNFR-associated death domain (TRADD)</td>
</tr>
<tr>
<td>Fas/CD95/Apo-1</td>
<td>Fas-associated death domain (FADD)</td>
</tr>
<tr>
<td>TRAIL receptor-1/Death receptor 4</td>
<td>FADD</td>
</tr>
<tr>
<td>TRAIL receptor-2/Death receptor 5</td>
<td>FADD</td>
</tr>
<tr>
<td>Apo-3/TRAMP/Death receptor 3</td>
<td>TRADD/FADD</td>
</tr>
</tbody>
</table>

Caspases are expressed as proenzymes (procaspases) comprising of a prodomain, p20 large, and p17 small subunits (Figure 1-7). The prodomain of the initiator caspases contains the sequences that are critical for the interaction of procaspases with other proteins. The initiator caspases have long prodomains. On the other hand, effector caspases have short prodomains. The short prodomains of the effector caspases are critical in preventing spontaneous activation of the effector caspase (146, 147). In the case of procaspases -8 and -10, the prodomain contains the death effector domain. In a
series of defined activation steps (148), the large and small subunits are separated (149). Then, the prodomain is removed.

**Figure 1-7. Caspase structure.** The prodomain is removed upon activation. The large (p20) and the small (p10) domains are the catalytic subunits. The active site cysteine residue lies in the p20 domain. The prodomain of initiator caspases includes sequences involved in interaction with other proteins These are the death effector domain (DED) and the caspase recruitment domain (CARD). Effector caspases have short prodomains.

Caspases are a class of cysteine proteases that are specific for substrates that have aspartic acid in the P1 position (i.e. N-terminal to the site of cleavage). One way to group caspases is by their function. Based on this criterion, caspases involved in apoptosis can be classified either as initiator/apical or effector/executioner caspases (Table 1-3). Initiator caspases catalyze their own activation (149) in response to apoptotic stimuli. Procaspases-8 and 10 are activated after sequestration in the DISC. A subset of the initiator caspases is also involved in inflammation processes. These, however, are beyond the scope of this dissertation. Upon activation, the initiator caspases cleave the effector caspases. Subsequently, the effector caspases cleave
different substrates. It is the proteolytic activity of the effector caspases that results in the morphologies that are associated with apoptosis.

**Table 1-3. The different caspases grouped according to their function in apoptosis**

<table>
<thead>
<tr>
<th>Initiator (plus involvement in inflammation)</th>
<th>Initiator/ Apical</th>
<th>Effector/ Executioner</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-1</td>
<td>Caspase-2</td>
<td>Caspase-3</td>
</tr>
<tr>
<td>Caspase-5</td>
<td>Caspase-8</td>
<td>Caspase-6</td>
</tr>
<tr>
<td>Caspase-11</td>
<td>Caspase-9</td>
<td>Caspase-7</td>
</tr>
<tr>
<td></td>
<td>Caspase-10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caspase-12</td>
<td></td>
</tr>
</tbody>
</table>

References: (150, 151)

Apoptosis is not only induced by extracellular stimuli. Apoptosis can also be initiated in response to stressors inside the cell, such as oncogene expression (152), DNA damage (153), hypoxia (154), and survival factor deprivation (155), in what is referred to as the intrinsic apoptotic pathway. In response to stress, cytochrome c is released from the mitochondria (156, 157). The transmission of the “internal stress” signal to the mitochondria is mediated by the Bcl-2 protein family. The Bcl-2 family can be grouped
into pro- and anti-apoptotic proteins. The ratio of the expression levels of these two groups plays a part in determining whether a cell will die (158).

The Bcl-2 protein family members contain one or more sequence homology domains called Bcl-2 homology domains (BH1, -2, -3, -4) (159) (Figure 1-8). These domains are involved in protein-protein interactions between the different Bcl-2 proteins. Aside from the BH regions, many of the Bcl-2 family members have a carboxy-terminal hydrophobic domain (referred to as TM in Figure 1-7). This domain can influence membrane localization (160). Different stimuli can result in altered expression or cellular localization of particular family members, and thus, either promote or inhibit apoptosis.

In response to specific intrinsic stresses, mitochondrial outer membrane permeabilization occurs and mitochondrial proteins are released, including cytochrome c. Apoptosis ensues (Figure 1-9). Mitochondrial outer membrane permeabilization results from formation of pores by two Bcl-2 family members, Bax and Bak. Bax is generally cytosolic. In response to apoptotic stimuli, it translocates to the cytosol, oligomerizes (161) and inserts into the mitochondrial membrane (162). In contrast, Bak resides in the mitochondria. It associates with another outer membrane protein, voltage-dependent anion channel 2 (VDAC2) (163). However, in response to apoptotic stimuli, this association is disrupted and Bak oligomerizes, as well (164). The Bax and Bak oligomers are presumed to comprise the mitochondrial apoptosis-induced channel
Anti-apoptotic Bcl-2 proteins: Bcl-2, Bcl-W, Bcl-xL, A1, Mcl-1

Pro-apoptotic Bcl-2 effector proteins: Bak, Bax, Bok

Pro-apoptotic BH3-only proteins: Bad, Bid, Bik, Bim, Bmf, Bnip3, Hrk, Noxa, Puma

Figure 1-8. Domain structure of Bcl-2 family members. The domain organization of the different groups of Bcl-2 family members is shown. The four Bcl-2 homology domains (BH1, -2, -3, and -4) are indicated, along with the transmembrane domain (TM). The pro-apoptotic Bcl-2 effector proteins are the proteins that have been shown to be directly involved in promoting mitochondrial outer membrane permeabilization (MOMP). Bok (Bcl-2-related ovarian killer protein) has not been shown to be involved in MOMP. However, it has the same organization as Bax and Bak.
Figure 1-9. Intrinsic activation pathway of apoptosis. In response to internal stressors, pro-apoptotic (e.g. Bad) and anti-apoptotic (e.g. Bcl-2) Bcl-2 protein family members regulate the mobilization of Bax (and Bak) to the mitochondrion. Bax oligomers form pores, resulting in mitochondrial outer membrane permeabilization. Cytochrome c (Cyt c) exits out of the cytoplasm through the Bax-formed pores. Cyt c promotes the oligomerization of Apaf-1. Apaf-1-oligomers in complex with cyt c recruit procaspase-9. Active caspase-9 is formed (aCaspase-9) and procaspase-3 is subsequently cleaved and activated (aCaspase-3). aCaspase-3 activity results in apoptosis (image courtesy of Walter J. Kline II).
(MAC). The MAC provides a transport pathway for cytochrome c across the outer membrane (165). Although Bax and Bak are the proteins directly involved in the mitochondrial outer membrane permeabilization, Bax and Bak activities are influenced by interactions with other Bcl-2 family members. There are two hypotheses that have been proposed to explain how the other Bcl-2 family members regulate Bax and Bak activity (Figure 1-10). In the direct activator-derepressor model, the BH3-only proteins are said to activate Bax and Bak. The anti-apoptotic proteins inhibit apoptosis by either keeping the BH3-only proteins from interacting with Bax and Bak (166), or by inhibiting activated Bax and Bak. In the indirect activator hypothesis, it is proposed that the anti-apoptotic Bcl-2 members interact with Bax and Bak. The BH3-only proteins compete with the anti-apoptotic proteins, possibly releasing Bax and Bak from their inhibitory interaction with the anti-apoptotic Bcl-2 members (167).

Cytochrome c is released with mitochondrial outer membrane permeabilization.

Cytochrome c interacts with the apoptotic protease activating factor 1 (Apaf-1) (168). Apaf-1 has three protein domains: the N-terminal caspase recruitment domain (CARD), the nucleotide-binding and oligomerization domain (NOD), and the C-terminal WD40-repeat domain. Cytochrome c interacts with the WD40-repeat domain. In the presence of dATP (169), oligomerization of the Apaf-1-cytochrome c complex occurs. The oligomer recruits procaspase-9 (170), forming a protein complex called the apoptosome. Apaf-1 and procaspase-9 interact via their caspase recruitment domains.
Direct activator-derepressor model

Inactive Bax/Bak + BH3-only → BH3-mediated activation → MAC

Indirect-Activator model

Indirect-Activator model

BH3

MAC is not formed

Figure 1-10. Hypothesis proposed to explain how the different Bcl-2 family members regulate the formation of the mitochondrial apoptosis-induced channel (MAC). In the direct activator-derepressor model, the pro-apoptotic BH3 members activate Bax and Bak, to form MAC. Cytochrome c (circles) are released. The anti-apoptotic Bcl-2 family members (hexagon) either bind to the BH3-only proteins or bind to the activated Bax/Bak, inhibiting MAC formation. In the indirect activator model, the anti-apoptotic Bcl-2 family members interact directly with Bax/Bak. The BH3-members do not directly associate with Bax/Bak. Instead, they sequester the anti-Bcl-2 family members, promoting MAC formation.
(CARD). Procaspase-9 is activated and procaspases-3 (170) and -7 (171) are subsequently cleaved.

Although there have been three effector caspases identified (Table 1-3), it has been shown that the primary effector caspase that is involved in causing many of the morphological changes associated with apoptosis is caspase-3 (172, 173). Caspase-3 is necessary for the nuclear condensation and DNA fragmentation seen in apoptosis, (172) at least in part, due to caspase-3 mediated activation of caspase-activated DNase (CAD) (174). While caspase-3 is the effector caspase that is most involved in apoptosis, caspases-6 and -7 also have important consequences. Caspase-6 cleaves Lamin A (172), a nuclear envelope protein. This is critical given that the breakdown of the nuclear envelope facilitates the entry of proteases and DNases. It has been shown that the caspase-6-mediated cleavage of Lamin A is necessary for the chromatin condensation and nuclear disassembly that occurs during apoptosis (175). Although caspase-3 cleaves more substrates than caspase-7, caspase-7 can be as effective as caspase-3 in terms of proteolytic activity on certain proteins, such as poly (ADP-ribose) polymerase (PARP) and inhibitor of caspase-activated DNase (ICAD) (173). The significance, however, of this redundancy in function remains to be established.
The intrinsic apoptotic pathway does not only involve caspases but also caspase-independent effectors. Caspase-independent effectors have been shown to induce apoptotic effects. These effectors are also released in response to mitochondrial outer membrane permeabilization. Two of these caspase-independent effectors are apoptosis-inducing factor (AIF) and endonuclease G. These two proteins move from the mitochondria to the nucleus. In the nucleus, they cause chromatin condensation and DNA fragmentation (176-178). The effects of AIF have been particularly important in apoptosis of neuronal cells (179, 180).

Apoptosis can be inhibited by endogenous mechanisms. The activity of caspases, and thereby, apoptosis, is negatively regulated by inhibitors of apoptosis (IAP). Caspase-8 activation can be inhibited by cIAP1 and cIAP2 (cellular Inhibitors of Apoptosis) (181). These IAP’s interact with the adaptor proteins associated with the death receptor TNF receptor -- TRAF1 and 2 (182). Another IAP, X-chromosome-linked Inhibitor of Apoptosis (XIAP), prevents cytochrome c-induced caspase cleavage. XIAP associates with caspase-9 and prevents caspase-9-mediated cleavage of caspase-3 (183, 184). XIAP can also bind and inhibit caspase-3 (185). Survivin is an IAP that is expressed in cancers but is rarely present in adult tissues (186). It interacts with XIAP and protects it from ubiquitin-dependent degradation (187). Thus, XIAP-mediated inactivation of caspases is prolonged.
The activity of XIAP can be counteracted by the protein Smac (Second mitochondria-derived activator of caspase)/ DIABLO (Direct Inhibitor of Apoptosis-Binding protein with LOw pl) (188). Smac is also a mitochondrial protein like cytochrome c (189). However, it has been shown that its release from the mitochondria occurs after caspase activation (190). Smac can interact with XIAP in at least two regions. XIAP has three baculoviral repeat (BIR) domains and a carboxy-terminal RING domain (191). The binding of Smac at the BIR3 region inhibits the interaction between XIAP and caspase-9 (192, 193). On the other hand, the binding of Smac to the BIR2 region sterically hinders the interaction of XIAP with caspases -3 and -7 (194, 195).

The intrinsic and extrinsic pathways of apoptosis are not entirely independent of each other (Figure 1-11). Cells have been classified into two types (Type I and II) based on the sufficiency of the response of their extrinsic apoptosis machinery, i.e. DISC and caspase-8, to induce apoptosis (196). In Type I cells, high caspase-8 activity is achieved with the initiation of the extrinsic apoptotic pathway. Thus, the extent of caspase-8 activation is sufficient to cleave caspase-3 and cause apoptosis. In the case of Type II cells, their response to death ligand in terms of caspase-8 activation is modest. Nevertheless, through the additional activation of the intrinsic pathway, apoptosis is enhanced. When these Type II cells are treated with Fas or TNFα, caspase-8 cleaves Bid, a pro-apoptotic Bcl-2 family protein. The cleaved (or what is referred to as truncated) Bid moves to the mitochondria and induces the release of cytochrome c. Thus, activation of the extrinsic pathway can result in the engagement of
Figure 1-11. Link between the extrinsic and intrinsic activation pathways of apoptosis. In Type II cells exposed to death ligand, activated caspase-8 can cleave the Bcl-2 family protein Bid. Truncated (tBid) moves to the mitochondrion and activates Bax, resulting in initiation of the intrinsic pathway of apoptosis. With cytochrome c, Smac is also released. Smac interacts with the inhibitor of apoptosis proteins (IAPs), releasing procaspase-3 and -9 from inhibitory interactions with the IAP's. Therefore, further caspase-3 activation and apoptosis ensues.

the intrinsic pathway, as well (170). It has been shown that Type II cells have less Fas receptor surface expression than Type I cells; hence, the lower levels of DISC formation (197) and resultant caspase-8 activity. Activation of the intrinsic apoptotic pathway can also affect the extrinsic pathway. As part of the initiation of the intrinsic pathway, SMAC is released. Consequently, the IAP-mediated inhibition on procaspase-3 is relieved. Therefore, caspase-8-induced cleavage of caspase-3 can ensue (193).

1.3.3 The need and the potential of targeting apoptosis in colon cancer therapy

Colon cancer cells have properties that inhibit apoptosis. The ability of colon cancer cells to resist apoptosis is correlated with chemoresistance to the chemotherapeutics 5-fluorouracil and oxaliplatin (198). Colon cancer cells and tissue have reduced expression of the death receptor Fas (199-201). Normal colon tissue expresses higher levels of Fas (202). In addition, decoy receptors are upregulated in colon cancer tissue (203, 204). Decoy receptors have similar extracellular, cysteine-rich, death ligand-binding domains as death receptors but decoy receptors do not have death domains.
They compete with the death receptors for death ligand binding without inducing apoptosis (205, 206). Therefore, the observed upregulation of the decoy receptors in colon cancer can explain in part why apoptosis of colon cancer cells is inhibited. As colon cancer cells metastasize, they acquire additional apoptosis resistance characteristics. In addition to a decrease in the Fas receptor of the extrinsic pathway (201, 207, 208), expression of the intrinsic apoptotic pathway protein Apaf-1 is also reduced. Moreover, expression of two members of the inhibitor of apoptosis (IAP) family, survivin and XIAP is elevated (207, 208). The downregulation of pro-apoptotic proteins and upregulation of anti-apoptotic proteins in colon cancer result in resistance to apoptosis.

Different strategies have been employed to induce apoptosis in colon cancer cells. One strategy is to downregulate anti-apoptotic proteins. Survivin is expressed in colon cancer cell lines and tissue (207-209). It is not detected (99, 186) or it is in low levels in normal colon (209). An analysis of survivin expression in colorectal carcinomas of colon cancer stages 0 to IV reveals that survivin-positivity is strongly associated with reduced apoptotic index (99). Downregulating survivin expression by siRNA has been effective in inducing apoptosis in colon carcinoma cells (210). Similarly, transfecting two colon carcinoma cell lines, SW480 and LOVO, with oncolytic adenovirus containing survivin shRNA increased apoptosis. Smaller tumors are formed in vivo when survivin is
knocked down (211). Livin, another member of the IAP family, can be downregulated by siRNA in HCT116 colon cancer cells. Consequently, apoptosis is increased in vitro. Moreover, intratumoral injection of the Livin siRNA results in decreased tumor growth (212). These studies demonstrate that promoting apoptosis in colon cancer by downregulating inhibitor of apoptosis proteins (IAP’s) can reduce tumor growth.

An alternative strategy to induce apoptosis is to employ pro-apoptotic proteins for therapy. Bik is one of the pro-apoptotic members of the Bcl-2 family. Overexpressing Bik in HT29 colon carcinoma cells increases apoptosis in vitro. Similarly, injecting adenoviral constructs of the Bik gene intratumorally to HT29 tumors induces apoptosis (213). The potential of pro-apoptotic proteins for colon cancer therapy have been taken to the clinic. Currently, there are two clinical trials exploring the use of the death ligand TRAIL in advanced stages of colon cancer (214). Aside from its inherent pro-apoptotic effect, TRAIL has been shown to affect cancer cells but not normal cells. This cancer-specificity is, in part, due to the upregulation of decoy receptors by normal cells (215). Because normal cells have higher levels of decoy receptors, they are not susceptible to TRAIL-induced apoptosis. Despite the efficacy of TRAIL, however, resistance mechanisms have been uncovered. TRAIL-resistant cells exhibit increased active caspase 8 degradation after TRAIL treatment (216). TRAIL-resistant cells can have lower caspase-8 levels (217). Thus, the TRAIL-induced activation of the caspase-8 apoptotic pathway is downregulated. Nevertheless, it has been shown that there is a potential for the use of pro-apoptotic proteins in colon cancer therapy. Key
considerations in their successful adaptation in the clinic are their effects on normal cells and possible resistance mechanisms. In the next section, the discovery of and the status of knowledge on another pro-apoptotic protein is discussed. This dissertation is focused on determining the pro-apoptotic activity of this protein and developing a strategy to increase its pro-apoptotic activity in colon cancer.

1.4 The pro-apoptotic protein Prostate Apoptosis Response Protein-4 (Par-4)

1.4.1 Initial characterization of Par-4

The pro-apoptotic protein, Par-4, was first identified in rat prostate cancer cells that were induced to undergo cell death. The upregulation of PAR-4 gene expression had occurred in response to intracellular calcium elevation but not to treatment with growth arrest or oxidative stress effectors (218). The human homolog of rat par-4 was identified in two independent experiments. In one, the goal of the study had been to elucidate protein-mediated regulation of the atypical isoforms of the enzyme protein kinase C (PKC), PKC ζ and PKC λ/1. Human Par-4 has been found to interact and regulate these two isoforms (219). In the other study, the human homolog of the rat par-4 gene was identified in a yeast two-hybrid screening (220) for proteins that interacted with the tumor suppressor and Bcl-2 regulator WT1 (221-223). The rat and human Par-4 protein sequences are 75% similar and 69% identical (Figure 1-12). The PAR-4 gene is ubiquitously expressed in normal human tissue types, including kidney, prostate and
Human 1  MATGGYRTSSGLGGSSTTDFLEEWKAKREKMRQNPAPPGGGSdakgpgagalgt  60
Rat 1  MATGGYRSS----STTDFLEEWKAKREKMRQNPVGCGSSGDPAAKSPACPLAQTTA  56
Human 61  paaaatelnnnlpggaapaavppgggvNCAVESAMLTSAAPCGRREDEppangasaa  120
Rat 57  AGTELNNHPGAAAAAPAPCGEALNCAHGSAT----PEAPCGSRREDECEIAGAAG  111
Human 121  ppocr-DEEPDGVPEKGSGPSARKGQIEKRKLREKRRSTGVVNIPAAECLDEYED  179
Rat 112  APASRDEEEDSAPPEKGSGPSARKGQIEKRKLREKRRSTGVVNIPAAECLDEYED  171
Human 180  DEAGQKERKREDAITQQNTIQNEAAPONLPGSYFFRPTVSRYKSTSVSEEDYSS  239
Rat 172  DEAGQKERKREDAITQQNTIQNEA5LPDPGTYSYLPDESRTPGRYKSTISAPEEELN  231
Human 240  RYSRTDSEPRYNDANVSGTLLVSSTLEKIEDLEKEVVERQENLRLVRIMQDKEEM  299
Rat 232  RYPRTDSEPSRNRTASPANFASSSTLEKIEDLEKEVLRERQENLRLTRIMQDKEEM  291
Human 300  IGKLKEIDLNLNRDLDEDENEQLQENKTLKVVGQLTR  340
Rat 292  IGKLKEIDLNLNRDLDEDENEQLQENKTLKVVGQLTR  332

Figure 1-12. Comparison of human and rat Par-4 sequences. Sequence alignment using the Basic Local Alignment Search Tool (225). Residues shaded in gray are identical, while residues shared in blue are similar residues in the human and rat sequences. Some of the sequences in the human Par-4 are presented in lower case letters. These are low complexity sequences that are filtered by BLAST to avoid artifactual matches (i.e. matches that do not really denote homology) that can occur with low complexity regions. The first underlined sequence denotes the SAC domain, and the second underlined sequence delineates the leucine zipper.
colon (220). The human Par-4 gene is located in chromosome 12q21 (224). Three Par-4 mRNA species have been identified from both human and rat tissues. They are 2.1, 5.0, and 7.3 kb in length, with the 2.1 kb being the most abundant mRNA (220).

1.4.2 Protein structure of Par-4

A number of protein domains have been identified in the the Par-4 protein (Figure 1-13). In its C-terminus, it has a domain (aa 258-340 in human sequence) that is homologous to the death domains of other proteins, e.g. Fas, TNF Receptor 1, Fas-associating protein with death domain (FADD) (219). The Par-4 death domain has been shown to be necessary and sufficient for the interaction of Par-4 with a nuclear proapoptotic factor THAP1 (226). However, it has not been conclusively shown that Par-4 interacts with other death domain-containing proteins, especially the death receptors. Although Par-4 may be a component of the death-inducing signaling complex (DISC) (at least in Jurkat cells), a direct interaction between Par-4 and either of the death-domain containing proteins in the DISC (Fas and FADD) has not been demonstrated (227).

The death domain of Par-4 lies within a putative coiled-coil region (specifically aa 262-340) (219, 229). A coiled coil is comprised of a protein sequence that has a heptad repeat (designated abcdefg), with hydrophobic residues occupying the “a” and “d” position. When a coiled coil has predominantly leucine at the “d” position, it is referred to as a leucine zipper. Par-4 contains a leucine zipper within its death domain. A leucine zipper (and coiled-coil) enables oligomerization of proteins. The polypeptide sequences
Figure 1-13. Protein domain organization of Par-4. Par-4 has a C-terminal leucine zipper (LZ) and a death domain (DD). The human Par-4 sequence has 342 amino acids. The death domain spans from aa 258-340. A Selective for Apoptosis induction in Cancer cells (SAC) domain has been identified and described in Par-4. The SAC domain has been shown to be sufficient to induce apoptosis (228). The two nuclear localization sequences, NLS1 and NLS2, and nuclear export sequence (NES) are shown.

that form the leucine zipper have an α helix conformation. The leucine residues of the helices comprising the zipper are concentrated in the space between the helices. This forms a hydrophobic region by virtue of the hydrophobicity of leucines (Figure 1-14).

The leucine zipper is found in many DNA-binding proteins. In addition, leucine zippers from different proteins can interact with each other via hydrophobic interactions, forming heterodimers (230). The leucine zipper plays a role in the activity of Par-4. Deleting the leucine zipper decreases Par-4 mediated apoptosis (228, 231-233). Thus, it can be surmised that at least part of the pro-apoptotic activity of Par-4 is dependent on the
Figure 1-14. An overhead view of a leucine zipper showing the positioning of the amino acid residues. The leucines are in position “d”. Position “a” is predominantly occupied by another hydrophobic residue, or leucine. Thus, the contact area of the two α helices of the leucine zipper is hydrophobic in nature.

function of the leucine zipper. There is a study, however, that has shown that the leucine zipper is not necessary for the pro-apoptotic action of Par-4 (228). The basis for this discordance remains to be elucidated. Transfecting a cell with the full length construct of Par-4 along with the leucine zipper abrogates Par-4 mediated apoptosis (231, 232). To explain these seemingly conflicting results, it has been proposed that the leucine zipper can function as a dominant-negative sequence. In some instances, overexpressing the leucine zipper is sufficient to decrease cell death in response to an apoptotic stimulus (233-235). Presumably, the leucine zipper is inhibiting the pro-apoptotic activity of endogenous Par-4 in these situations. It is possible that the leucine zipper competes with full-length Par-4 in binding ligands, including other proteins. The interaction of full-length Par-4 with other ligands and/or proteins may be critical in the
pro-apoptotic activity of Par-4. A number of proteins interact with Par-4 via the leucine zipper domain, e.g. WT1 (220), Akt1 (236), DAP-like kinase (Dlk) (237), the atypical PKC-interacting protein p62 (238), apoptosis-antagonizing transcription factor (239), Amida (240), DNA topoisomerase 1 (241). In many of these interacting proteins, the Par-4 leucine zipper is not interacting with a specific motif. However, in a few of these interacting proteins, specifically WT1 (220) and atypical protein kinase C's (219), the leucine zipper of Par-4 interacts with zinc finger domains.

Par-4 has two nuclear localization sequences (NLS1 and NLS2) and one putative nuclear export signal (NES). NLS1 is at amino acids 24-29 and NLS2 is at amino acids 145-161. Deletion of NLS1 decreases nuclear localization only by 7-11%, while deletion of NLS2 decreases nuclear localization by 85% (228). In addition, it has been shown that a Par-4 construct that does not have the first 68 amino acids is still effective in inducing apoptosis. This indicates that NLS1 is not necessary for the apoptotic activity of Par-4 (242). On the other hand, a deletion construct of NLS2 is ineffective in causing cell death. This result led to the proposal that NLS2 is necessary for Par-4 mediated apoptosis (228). However, this result is not conclusive given that adjacent to NLS2 is a phosphorylation site that has been shown to be essential for Par-4 activation, Thr163. Thus, the deletion of the NLS2 could have disrupted the site for this essential activating phosphorylation of Par-4. The disruption of Par-4 mediated apoptosis in response to the NLS deletion may not have been because NLS2 is essential but because the activation of Par-4 has been inhibited with the deletion. This issue can be addressed in part by
studying the effects of mutating NLS2 on Par-4 mediated apoptosis. The putative NES of Par-4 is at amino acids 301-312 in the human sequence (228). Its actual function, however, has not been studied.

A domain that is sufficient to cause apoptosis in cancer cells and not in normal cells has been identified in Par-4. The SAC (selective for apoptosis induction in cancer cells) domain includes aa 146 to aa 200. It is a region that is 100% conserved in the rat and human sequences (228). Similar to results observed with the full-length Par-4 sequence, injecting subcutaneous tumor xenografts with an adenoviral construct for SAC results in reduced tumor growth. SAC transgenic mice have been developed. They have increased resistance to spontaneous tumor growth (243). Overexpression of the SAC domain results in apoptosis in cancer cells but not in normal cells (228). Given the pro-apoptotic activity and cancer-selectivity of the SAC domain, efforts have been made to increase its applicability for therapy. A genetic construct has been made that encodes a fusion protein comprised of a synthetic signal peptide [NT4(Si)], the Par-4 SAC domain, the N-terminal sequence of the influenza virus hemagglutinin protein (HA2) and the Tat peptide sequence (i.e. NT4(Si)-Par-4 SAC-HA2TAT) (244). The Par-4 SAC construct was delivered into cells using an adeno-associated virus vector. Adeno-associated viral vectors have been shown to facilitate long-term gene expression (245, 246). This Par-4 SAC construct is effective in causing apoptosis (244).
The C-terminal half of Par-4 contains protein domains and structural motifs that are involved in protein function, as has been discussed. Nevertheless, part of the Par-4 sequence is predicted to be disordered (229), based on DisEMBL analysis. DisEMBL is a computational tool for prediction of disordered/unstructured regions within a protein sequence. One reason for the identification of disordered sequences in a protein is the observation that disordered protein regions actually serve as sites of multiple interactions with other proteins (247). When Par-4 is subjected to limited proteolysis with trypsin, 80% of it is degraded. In contrast, a folded protein, bovine serum albumin is only 10% degraded under the same conditions. Given that a folded protein is less susceptible to proteolysis, the susceptibility of Par-4 to degradation is an indication of its unstructured nature. The amino acid sequence (at least) of rat Par-4, is characteristic of a class of proteins called intrinsically disordered proteins (IDP) (229). Intrinsically disordered proteins lack aromatic residues and have an excess of hydrophilic amino acids (248, 249). Specifically, they have a higher proportion of arginine, lysine, asparagines, proline, and serine, and a lower proportion of cysteine, tryptophan, tyrosine, isoleucine, and valine, when compared with ordered proteins (250). Because intrinsically disordered proteins lack hydrophobic residues, they do not form a hydrophobic protein core and a stable tertiary structure. This does not mean, however, that intrinsically disordered proteins, including Par-4, exist as unfolded strings of amino acids. What may occur instead is that these proteins form different conformations in a transient manner (229). This could explain how Par-4 and other intrinsically disordered proteins can have a myriad of interactors.
1.4.3 The role of Par-4 in apoptosis in different cell types

The role of Par-4 in apoptosis is evident at the onset, given that it was first identified in cells exposed to apoptotic stimuli (218). In normal tissues, increases in Par-4 expression are detected in cells undergoing apoptosis, e.g. prostate ductal epithelial cells under conditions of testosterone ablation and atretic follicles of the ovary (251). Par-4 expression has been found to be necessary for apoptosis to occur in certain cell types. In AT-3 prostate cancer cells, Par-4 expression is necessary for apoptosis in response to the ER stressor thapsigargin. Thapsigargin treatment results in increased Par-4 expression. Using an antisense oligonucleotide, this increase in expression was abated. The inhibition of Par-4 expression was correlated with a reduction in thapsigargin-induced apoptosis (231). Par-4 has also been shown to be necessary for neuronal apoptosis in response to a myriad of conditions, including glutamate and staurosporine treatment (252), trophic factor withdrawal (253), FeSO₄ (induces production of hydroxyl radicals and causes membrane lipid peroxidation in neurons) and hydroxynonenal (a lipid peroxidation product) addition (254); transient ischemia; and sodium cyanide (an inducer of chemical hypoxia) treatment (255). In these studies, it has been shown that Par-4 elevation occurs before significant changes in apoptosis parameters, such as caspase activity or extent of mitochondrial dysfunction, are detected. Thus, it can be surmised that Par-4 is involved in the induction of apoptosis. However, the question remains on how such a wide variety of apoptotic stimuli can result in similar upregulation of Par-4 and apparent consequential apoptosis. One possibility is that many, if not all, of these stimuli cause disruption of cellular calcium
homeostasis and oxidative stress (253, 256, 257). These two factors have been shown to result in Par-4 expression (218, 252).

Par-4 has been shown to be essential for apoptosis under a myriad of conditions, as previously discussed. It is, however, downregulated in a number of cancers, e.g. renal (258), cholangiocarcinoma (259), pancreatic (260), endometrial (261), and lung (262). Thus, the effects of overexpressing Par-4 to induce apoptosis have been investigated. These studies have shown that Par-4 has cell type-specific effects. There are cell types that are susceptible to Par-4 overexpression alone. Par-4 overexpression is sufficient to induce apoptosis in a subset of cell lines -- breast cancer (243, 263), androgen-independent prostate cancer (236, 243, 264), the androgen-dependent prostate cancer cell line TRAMP, lung cancer, cervical cancer (243) and nasopharyngeal cancer (265). In vivo, Par-4 overexpressing melanoma cells form tumors that have a significantly higher apoptosis rate (266). A consequence of this increase in apoptotic rate is a smaller tumor size. Par-4 overexpression can be induced in vivo by injecting subcutaneous xenografts of prostate cancer cells in mice with adenoviral construct of Par-4. The increased expression of Par-4 has been sufficient to inhibit tumor growth of these prostate cancer xenografts (264). In other cell lines, Par-4 overexpression is not enough to increase apoptotic rates e.g. Jurkat T lymphocytes (267), androgen-dependent prostate cancer cell line, LNCaP (236, 264), melanoma cells (231), and renal carcinoma (268). In these cells, however, increased Par-4 sensitizes the cells to apoptotic stimuli (231, 267, 268), including UV irradiation and serum-withdrawal (219).
Among the pro-apoptotic agents that have been tested are agents that are used in the clinic, doxorubicin (258), camptothecin (269), and ionizing radiation (270).

1.4.4 The impact of Par-4 on the extrinsic and intrinsic pathways of apoptosis

The pro-apoptotic activity of Par-4 involves both the extrinsic and intrinsic pathways of apoptosis. Par-4 induced apoptosis can be blocked by a Fas ligand decoy, Fas-Fc, in prostate cancer cells. Fas-Fc has the extracellular domain of the Fas receptor but does not contain a death domain. It can interact with FasL without causing apoptosis. Thus, the decoy inhibits Fas/FasL-initiated apoptosis. Other strategies to block the extrinsic pathway, use of a Fas antagonist decoy, transfection with dominant negative-Fas-associated death domain (FADD) and dominant negative caspase-8, are also effective in inhibiting Par-4 mediated apoptosis. This indicates that Par-4 induced apoptosis involves the extrinsic pathway (264).

Par-4 activates the extrinsic pathway of apoptosis via a number of mechanisms (Figure 1-15). Par-4 overexpression has been shown to result in mobilization of the Fas death receptor in malignant lymphocytes (271). In the prostate cancer cell line, both Fas death receptor and FasL are mobilized to the cell surface. Consequently, interaction between the Fas death receptor and FADD is increased (264). The surface expression of another death receptor, DR5, is upregulated in Par-4-overexpressing renal cancer cells that have been treated with the death ligand TRAIL (233). Consequent to the increased
Figure 1-15. Mechanisms by which Par-4 promotes the extrinsic pathway of apoptosis. I. Par-4 increases mobilization of Fas death receptor. II. Par-4 promotes localization of the Fas death ligand to the membrane. III. Par-4 functions as a death ligand, interacting with GRP78 in the membrane. This results in activation of FADD and caspase-8. IV. Par-4 and GRP78 are necessary for apoptosis in response to the death ligand TRAIL.
activation of the extrinsic pathway of apoptosis by Par-4 (whether through Fas or TRAIL), caspase-8 cleavage is increased (233, 264). Par-4 can also function like the extracellular death ligands and cause caspase-8 activation. Extracellular Par-4, however, does not interact with the death receptors. Instead, it interacts with another receptor protein, GRP78. The Par-4-GRP78 interaction results in FADD-mediated recruitment and activation of caspase-8 (272). The mechanism behind FADD activation by GRP-78 is not known. GRP78 does not have a known death domain that can mediate an interaction with FADD.

The Par-4 protein itself has a death domain (219). It has been found to interact with Fas, as part of the death-inducing signaling complex (DISC) in Jurkat cells. After caspase-8 is cleaved, a decrease in Par-4 level in the DISC is observed (227). The significance of the apparent exit of Par-4 from the DISC upon caspase-8 cleavage, and the role that Par-4 plays in the DISC remain to be elucidated. Whether Par-4 is part of the DISC in all cancer cell types has not been established.

Par-4 also impinges on the intrinsic pathway of apoptosis. Par-4 overexpression can downregulate the expression of Bcl-2 (267, 270, 273-275), resulting in caspase-9 activation. The importance of this effect of Par-4 on Bcl-2 is demonstrated by the observation that overexpressing Bcl-2 is sufficient to counteract Par-4 induced apoptosis in response to growth factor deprivation (242). In vivo, an inverse correlation
has been found between Par-4 and Bcl-2 expression in prostate cancer tissue (274), in acute lymphocytic leukemia cells, and in lymphocytic cell lines (276). The inverse relationship between Bcl-2 and Par-4, however, is not observed in all cell types. In androgen-independent prostate cancer cells, despite overexpression of Bcl-2, Par-4 is still able to induce cell death (264).

The transcription of Bcl-2 is regulated via two promoters, the major P1 and the minor P2. The P1 promoter, the predominantly used promoter, has no TATA box. This enables multiple sites of transcription initiation. It is located approximately 1.7 kbp upstream of the open reading frame. Transcription from this promoter yields an mRNA that has a long 5’ untranslated region. The P2 promoter is used in expression of only 5% of bcl-2 transcripts. It is located approximately 80 bp upstream of the open reading frame. The promoter has a TATA and a CCAAT box. Thus, transcription initiation occurs in two defined sites (277). Bcl-2 expression is regulated by a number of proteins. The tumor suppressor p53 can downregulate Bcl-2 expression. A negative response element has been identified in the Bcl-2 gene that is responsive to p53 levels (278). Other negative response elements, referred to as the \( \pi \) 1 sequences serve as the binding site of an Ets family protein. These sequences are used to negatively regulate Bcl-2 expression in pre-B lymphocytes (279). The expression of Bcl-2 can also be positively regulated. The NFkB p50 subunit has been found to bind a site in the P2 promoter. The transcriptional activity from the Bcl-2 P2 promoter is increased by NFkB, especially in response to TNF\( \alpha \) treatment (280).
The Par-4 interacting protein, WT1 regulates Bcl-2 expression. WT is a zinc finger transcription factor. It can function as either a transcriptional activator or repressor (281, 282). Its effect on gene expression is dependent in part on the position of a consensus binding site (GNGNGGGNG) relative to the transcriptional start site. The WT1 protein contains an activation and a repression domain, enabling it to regulate gene expression positively or negatively (282). WT1 can upregulate Bcl-2 expression in rhabdoid tumor, osteosarcoma and monkey kidney cell by binding to a positive control element (5’-GCGTGGGAGT-3’) in the P1 promoter. This positive control element is at -1807 from the transcriptional start site of the Bcl-2 gene (223). On the other hand, WT1 negatively regulates Bcl-2 expression via its interaction with a site -1406 from the transcriptional start site of the Bcl-2 gene (283). Par-4 exists in a complex with WT1 at this site. The Par-4 leucine zipper interacts with the zinc finger domain of WT1. The WT1-mediated transcriptional repression of Bcl-2 expression is promoted as Par-4 levels are increased (275). It has been proposed that Par-4 has its own repression domain that contributes to the inhibition of gene expression.

Par-4 promotes apoptosis by downregulating inhibitor of apoptosis proteins (IAP). Knocking out Par-4 has been shown to result in upregulated expression of the X-linked inhibitor of apoptosis. Par-4 overexpression also results in increased levels of the caspase-independent effector AIF (271). There have been two reports that Par-4 also increases the expression of procaspase-9 (271, 284). However, the results presented
are not conclusive. Nevertheless, the mechanisms by which Par-4 regulates the expression of the XIAP and AIF remain to be elucidated.

1.4.5 Interactions of Par-4 with pro-survival proteins

Par-4 does not only directly impinge on the players of the pro-death pathway, but it also affects the pro-survival proteins NFκB, PKCζ and Akt1. Similar to the case with the apoptotic pathways, the pro-survival pathways are also linked. Although PKCζ and Akt have their specific effects on apoptosis, both proteins increase survival by promoting NFκB activation. PKCζ also activates Akt (285).

One of the first protein interactions of Par-4 that has been reported is its interaction with the atypical isoforms of protein kinase C (PKC), PKCλ/ and PKCζ. The leucine zipper of Par-4 interacts with the zinc finger domains of atypical PKCs (219). PKC is a lipid-regulated serine/threonine kinase. The isoforms of PKC are divided into three classes based on an isoform’s dependence on diacylglycerol and Ca$^{2+}$ for their activity. All protein kinase C isoforms require phosphatidylserine. The classical or conventional isoforms include PKC α, βI, βII, and γ. They require Ca$^{2+}$ and diacylglycerol/phorbol ester (286). On the other hand, the novel isoforms (δ, ε, η/(L), θ, and μ) do not require Ca$^{2+}$ for activation (287, 288). The atypical protein kinase C isoforms are PKC λ/ and ζ (289). They do not require Ca$^{2+}$ or diacylglycerol (290, 291). Nevertheless, they are regulated by other lipid cofactors, namely, phosphatidylinositol 3,4,5-trisphosphate and
ceramide (291, 292). The different PKC isoforms each have their own effects on apoptosis. Given that the atypical PKC's are the isoforms that interact with Par-4, only their pro-survival role will be discussed.

Par-4 interacts with the atypical PKC's, PKCλ/γ and PKCζ (219, 238). PKCζ kinase activity (238) is inhibited by Par-4. It is proposed that the binding of Par-4 to PKCζ involves a conformational change in the kinase that results in inhibition of its activity. The inhibition of PKCζ is one of the mechanisms behind the pro-apoptotic activity of Par-4. PKCζ inhibits Fas-mediated apoptosis in the promyeloblastic cell line KG1a by inhibiting the recruitment of procaspase-8 to the DISC. PKCζ interacts with and phosphorylates FADD. When PKCζ is downregulated by overexpressing Par-4, the effects of PKCζ on FADD phosphorylation, DISC formation, and caspase-8 activity are abrogated (293). It is not yet known, however, how the phosphorylation of FADD by PKCζ results in inhibition of the recruitment of procaspase-8 to the DISC.

The more studied mechanism by which PKCζ inhibits apoptosis is its activation of another pro-survival protein NFκB (Figure 1-16). NFκB (nuclear factor κB) is a transcription factor (294, 295) that promotes cell survival by inducing the transcription of genes coding for anti-apoptotic proteins like Bcl-xL (296), and TNF-receptor associated factors (TRAF1 and TRAF2), c-IAP1, c-IAP2 (181), and c-FLIP (297). NFκB recognizes decameric sequence motifs (called κB elements) in the promoters and
enhancers of the genes it regulates. The κB consensus sequence is 5'-GGGRNYYYCC-3. R refers to any purine and Y refers to any pyrimidine. The most abundant form of NFκB is a heterodimer of p50 (48-51 kD) and RelA/p65 (65-68 kD) protein subunits. Both subunits contain a highly conserved DNA-binding/dimerization domain called Rel homology domain (RHD) (298). NFκB is basally kept inactive in the cytoplasm by its interaction with IκB (299-301). In response to a stimulus, IκB is phosphorylated by IκB kinase (IKK) (302), and targeted for degradation by the proteasome. This exposes the

![Diagram of NFκB activation](image)

**Figure 1-16. Activation of NFκB by PKCζ.** PKCζ phosphorylates IκB kinase (IKK), activating it. IKK phosphorylates IκB, targeting its for degradation by the proteasome. With the degradation of IκB, the p50/p65 NFκB dimer enters the nucleus and activates the transcription of its target genes.
nuclear localization sequence in the p65/RelA subunit of NFκB, enabling the dimer to move to the nucleus. In the nucleus, the dimer recognizes the genes that contain the κB elements and activates the expression of these genes. PKCζ promotes NFκB-mediated transcription by interacting with, phosphorylating and activating one of the IκB kinases, IKKβ (303). The activated IKK phosphorylates IκB. Subsequently, IκB is ubiquitinated and targeted for degradation by the proteasome. Thus, mobilization of the NFκB dimer and the ensuing NFκB-mediated transcription is increased.

The serine/threonine kinase Akt inhibits cell death as a result of its phosphorylation of proteins involved in apoptosis. These include the pro-apoptotic protein Bad, procasapse-9, and the Forkhead transcription factor FKHR1/FOXO3A. Akt1 phosphorylates the BH3-only Bcl-2 family member Bad at a serine site (304). Phosphorylated Bad interacts with the scaffolding protein 14-3-3. Consequently, Bad is kept from heterodimerizing with the anti-apoptotic proteins Bcl-2 and Bcl-xL. When Bad is unphosphorylated, it interacts with Bcl-2 and Bcl-xL. These keep Bcl-2 and Bcl-xL from associating with Bax (Figure 1-10). As a result of phosphorylation of Bad by Akt, Bcl-2 and Bcl-xL are available to heterodimerize with Bax. This inhibits Bax-mediated formation of mitochondrial pores as part of the activation of the intrinsic pathway of apoptosis. Thus, Akt phosphorylation of Bad essentially inhibits the engagement of the intrinsic pathway of apoptosis. Procasapse-9 is also phosphorylated by Akt at a serine site. Although it has been shown that this phosphorylation inhibits caspase-9 activity (305) and hence, apoptosis, the mechanism behind how caspase-9 phosphorylation
alters activity has not been determined. Akt does not only phosphorylate proteins that are directly involved in apoptosis. FKHRL1/FOXO3A is a transcription factor that may regulate transcription of the gene for Fas L, an initiator of the extrinsic pathway of apoptosis. When Akt phosphorylates FKHRL1/FOXO3A, FKHRL1/FOXO3A also interacts with the scaffolding protein 14-3-3 (similar to Bad). The interaction of the transcription factor with 14-3-3 results in its sequestration in the cytoplasm. Thus, it is unable to upregulate Fas L expression (306).

Akt has also been shown to activate NFκB (similar to PKCζ). Just like PKCζ, Akt activates an IKK. However, in contrast to PKCζ which activates IKKβ, Akt activates both IKKα and IKKβ. IKKβ is the IKK primarily involved in phosphorylating IκB and targeting it for degradation, especially in response to inflammatory stimuli like TNFα (307). On the other hand, IKKα and IKKβ are involved in phosphorylation (308, 309) and activation of the p65 NFκB subunit (310). This phosphorylation is necessary for NFκB-mediated gene transcription, at least in response to inflammatory stimuli like TNFα (310). Therefore, Akt, through its activation of both IKK α and IKKβ, increases phosphorylation and activation of p65.

Par-4 has been shown to be involved in regulating Akt1 phosphorylation. In tissues of Par-4 knockout mice, Akt1 phosphorylation at Serine 473 site (262, 311) and Akt1 kinase activity are elevated (262). This suggests that Par-4 regulates Akt1, reducing
Akt1-mediated phosphorylation and inhibition of pro-apoptotic proteins. Overexpressing PKCζ reversed the effect of Par-4 on Akt. PKCζ phosphorylates Akt at Ser124. The phosphorylation of Ser124 by PKCζ and the phosphorylation of Thr450 by Janus-like kinase (JNK) (262, 285) are necessary for activation of Akt (312). This indicates that what appears to be an effect of Par-4 on Akt1 may actually be a consequence of the inhibitory interaction of Par-4 with PKCζ. This inhibitory interaction of Par-4 on PKCζ may result in reduced phosphorylation of Akt by PKCζ on the Ser124. This leads to reduced phosphorylation at Ser473 and inhibition of Akt kinase activity. However, the possibility that Par-4 inhibits Akt1 directly cannot be discounted. Akt1 has been shown to interact and phosphorylate Par-4 (236). Thus, it is not improbable that Par-4 can also inhibit Akt1.

Par-4 downregulates the NFκB pathway partly by inhibiting PKCζ and Akt. Oncogenic Ras and activated Raf-1 (downstream effector of Ras) proteins increase NF kappa B activity (313), thereby, inhibiting apoptosis. Overexpressing Par-4, however, abrogates this effect of Ras on apoptosis. Par-4 inhibits the Ras-induced activation of NFκB activity (314). Par-4 may not only inhibit NFκB by inhibiting PKCζ, Akt, and Ras. There is also a possibility that Par-4 directly interacts with NFκB (315).
Given that Par-4 inhibits PKCζ and Akt, proteins that both upregulate NFκB activity, it is not surprising that inhibition of NFκB activity is one of the important consequences of Par-4 activation. Par-4 overexpression inhibits the DNA-binding (270) and transcriptional activity of NFκB (228, 264), including the NFκB-induced transcription of Bcl-2 (270). When Par-4 expression is knocked down in mice, increased localization of the NFκB p65 subunit to the nucleus occurs (262, 311). The importance of inhibition of NFκB in Par-4 mediated apoptosis can be gleaned from the observation that Par-4-induced apoptosis in prostate cancer cells is abrogated by overexpression of the p65 subunit (264). Par-4 has been shown to prevent the IκBα phosphorylation that occurs in response to radiation (270). However, the mechanism behind how Par-4 inhibits the radiation-induced activation of the NFκB pathway is yet to be elucidated.

1.4.6 Regulation of Par-4 expression and activity

Par-4 levels are regulated by mechanisms that have yet to be fully elucidated. One mutation has been detected in an endometrial carcinoma tissue sample (311) among 69 samples. This mutation resulted in a deletion of the COOH-terminal half of Par-4. Par-4 gene expression can also be downregulated by promoter methylation. This has been observed in endometrial and lung cancers (261, 311). Promoter hypermethylation is a mechanism used by cells to inhibit gene expression. It is catalyzed by enzymes called DNA methyltransferases. Oncogenic Ras can increase the methylation of the Par-4 promoter (316). Ras, through its downstream effector mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK), has been shown to
upregulate the expression of DNA methyltransferase (Dnmt) 1 and 3a. Dnmt1 has a higher affinity for hemimethylated (i.e. methylated on one strand) over unmethylated DNA (317, 318). Therefore, it maintains DNA methylation patterns. On the other hand, Dnmt3a acts on unmethylated DNA. Although Ras upregulates the expression of both Dnmt1 and 3a, it is not certain which Dnmt actually methylates the Par-4 promoter.

Another oncogene, v-Src, has also been shown to increase the methylation of the Par-4 promoter, at least in fibroblasts. V-Src was shown to increase the expression of DNA methyltransferase 1. Par-4 promoter hypermethylation is attributed to the increase in Dnmt1. Levels of the other Dnmt’s have not been reported in these v-Src-overexpressing cells (319).

Par-4 gene expression can be upregulated by NFκB. This has been observed in endometrial carcinoma cells. The PAR-4 promoter has three putative NFκB consensus binding sequences, to which the NFκB p65 subunit can bind (320). This seems counterintuitive, given that NFκB as previously discussed upregulates the expression of anti-apoptotic proteins. However, it has been shown that NFκB can activate the expression of pro-apoptotic proteins under certain conditions. In T lymphocytes, expression of the death ligand Fas L is upregulated by NFκB in response to T cell receptor activation (321).
There is evidence that Par-4 is post-transcriptionally regulated. Discordance in the patterns of mRNA and protein expression has been observed in a number of systems. In the normal endometrium, Par-4 mRNA was decreased in stroma of late secretory epithelial cells in comparison to that in proliferative epithelial cells. On the other hand, Par-4 protein was observed to be higher in the stroma of these late secretory epithelial cells (320). This discordance in Par-4 mRNA and protein expression has also been reported in mononuclear cells from healthy volunteers and chronic lymphocytic leukemia patients (CLL). In 10 samples from healthy patients, no Par-4 mRNA was detected. However, Par-4 protein was expressed in all of the samples. In the case of the CLL patients, 11 out of 30 samples did not have detectable Par-4 mRNA. On the other hand, all of the samples had high Par-4 protein levels (276). The translational control of Par-4 is poorly studied. One protein that has been shown to inhibit the translation of Par-4 mRNA to protein is the latent membrane protein of the Epstein-Barr virus. However, the mechanism of this inhibition has not been elucidated (322).

The pro-apoptotic activity of Par-4 is influenced by its phosphorylation state. Par-4 is phosphorylated by protein kinase A (PKA) on the threonine 163 site (Thr155 in rat sequence). Mutating this site abolishes the pro-apoptotic activity of Par-4. Two different kinases have been shown to phosphorylate this site, DAP-like kinase/zipper-interacting protein kinase (Dlk/ZIP) (323), and protein kinase A (PKA) (263). It is possible that Par-4 is phosphorylated by a particular kinase depending on the cell type. Dlk/ZIP has been shown to be functional in rat fibroblasts while PKA has been studied in cancer cells.
Given that the focus of this dissertation is primarily on cancer, PKA will be mentioned more often in association with Par-4 phosphorylation at this site. Nevertheless, it is important to point out that another kinase may be acting on Thr163 especially in different cell types.

Endogenous Par-4 is phosphorylated at Thr163 in cancer cells, but not in normal cells. The higher PKA activity of cancer cells in comparison to normal cells has been proposed as the mechanism behind the sensitivity of cancer cells to the effects of Par-4 (263). Protein kinase A or what is also referred to as cyclic AMP-dependent protein kinase, is generally activated upon binding of cyclic AMP. Nevertheless, PKA can be activated in a cAMP-independent manner. The catalytic subunit of PKA has been shown to interact with IκB. When IκB is activated by IKK and targeted for degradation, the PKA catalytic subunit is also released (along with the NFκB dimer) and activated (324). PKA recognizes the consensus sequence RRXS/TB (with X indicating any possible amino acid and B specifying a hydrophobic residue) (325, 326) and phosphorylates its substrates at serine or threonine residues. PKA has two isoforms, PKA-I and PKA-II. Although PKA-II is constitutively expressed in normal differentiated cells, PKA-I is only transiently overexpressed when normal cells are induced to proliferate. PKA-I has been found to be overexpressed in cancers. This overexpression has been correlated with worse clinical and/or pathological features in colon, breast, and ovarian cancer (327-329). PKA enzymatic activity has also been found to be elevated in cancers (263). Although phosphorylation of Par-4 by PKA is necessary, it is not sufficient to induce
Par-4 mediated apoptosis. It has been found that increasing PKA activity in normal cells that have endogenous Par-4 is not sufficient to induce apoptosis. Increased PKA-mediated phosphorylation of Par-4 has to be combined with an apoptotic stimulus to cause cell death in normal cells.

Most cells express Par-4 endogenously. Cells can be protected from Par-4-induced apoptosis by a mechanism that keeps Par-4 inactive. One such mechanism is mediated by Akt. Par-4 is phosphorylated by Akt at a serine site (serine 249 in rat sequence). One consequence of this phosphorylation is the interaction of Par-4 with the scaffolding protein 14-3-3. This association promotes the sequestration of Par-4 in the cytoplasm (236). The localization of Par-4 in the cytoplasm is critical given that the pro-apoptotic activity of Par-4 has been correlated with its localization to the nucleus (228). This brings us to another putative mechanism of Par-4 regulation -- subcellular localization.

The pro-apoptotic activity of Par-4 has been correlated with its subcellular localization in a particular cell type. Cells that have endogenous or overexpressed Par-4 in the cytoplasm are not susceptible to Par-4-mediated apoptosis. Par-4 overexpression has to be combined with another apoptotic stimulus to increase cell death in these cells. On the other hand, other cell types have Par-4 in their nuclei. These undergo cell death when Par-4 is overexpressed (228). The localization of Par-4 is expected to be partly determined by its nuclear localization sequences (NLS) and nuclear export sequence.
Its first NLS sequence, NLS1, is in positions 24-29. It is not necessary for Par-4 mediated apoptosis (228, 242). On the other hand, the second NLS sequence, NLS2 (at amino acids 145-161) has been proven to be a functional NLS. The SAC domain, the domain of Par-4 that is sufficient in itself to cause apoptosis, includes NLS2 (Figure 1-12). The NLS2 imparts on Par-4 and SAC the ability to be localized to the nucleus. Par-4 has a putative NES at amino acids 301-312 in the human sequence (228). Currently, little has been published on the role of the NES. Nevertheless, there is evidence that Par-4 nuclear export is significant to Par-4 function. Par-4 interacts with another pro-apoptotic protein DAP-like kinase (Dlk)/zipper-interacting protein kinase (ZIP). Dlk is predominantly in the nucleus. However, through its interaction with Par-4, Dlk is mobilized to the cytoplasm, at least in rat fibroblasts. The cytoplasmic localization of Dlk has been correlated with its pro-apoptotic activity (237, 330). This indicates that Par-4 can move out of the nucleus foreseeably through its NES.

An effector of K-Ras, RASSF2, has been shown to be necessary for mobilization of Par-4 to the nucleus in response to TRAIL treatment or activated K-Ras overexpression (331). RASSF2 is a nuclear protein. It contains a nuclear localization signal (NLS). The localization of RASSF2 in the nucleus is critical given that deletion of its NLS diminishes the inhibitory effect of RASSF2 on tumor growth (332). RASSF2 has been implicated in nuclear transport. It has an arginine-rich sequence (that is also in RASSF1C and RASSF5) by which RASSF2 interacts with the nuclear transport receptor, importin-α. Consequently, proteins interacting with RASSF2 are transported into the nucleus along
with RASSF2 (333). It is therefore highly possible that Par-4 interacts with RASSF2 and consequently, gets mobilized to the nucleus as a result of this interaction. Par-4 has been observed to move to the nucleus in response to TRAIL (334) and apogossypolone (a small molecule inhibitor of the Bcl-2 family members) (335, 336). The question of whether these stimuli promote Par-4 movement to the nucleus through the action of RASSF2 (and the Ras pathway) remains to be pursued.

1.5 Dissertation Objectives

From the body of work that has been presented here, the following conclusions can be made:

1. There is a need to develop more effective therapies for colon cancer, especially with minimal side effects.

2. There is a need and potential in targeting apoptosis as a strategy in colon cancer therapy.

3. Prostate apoptosis response protein-4 (Par-4) is involved in apoptosis induction in different contexts.

These findings have led our research group to pursue the long-term goal of developing treatment regimens for colon cancer that exploit the pro-apoptotic activity of Par-4. However, because at the beginning of this dissertation work, only one report of the
involvement of Par-4 in colon cancer has been made (337), the groundwork had to be laid in establishing the apoptotic activity of Par-4 in colon cancer. I hypothesize that increasing the pro-apoptotic activity of Par-4 in colon cancer results in cell death.

To pursue this hypothesis, I formulated two specific aims:

1. Determine the impact of overexpressing Par-4 on cell death in colon cancer.

2. Elucidate how endogenous Par-4 can be activated in colon cancer, resulting in cell death.
Chapter 2

Par-4 promotes Apoptosis in colon cancer cells

2.1 Introduction

The impact of overexpressing Par-4 on cell death in colon cancer has not been explored. When this dissertation work was started, there had been only one report of a study of Par-4 involvement in colon cancer. In this study, HCA-7 colon carcinoma cells were treated with cyclooxygenase (COX) inhibitors/nonsteroidal anti-inflammatory agents (NSAID's) (337). COX inhibitors are currently tested as chemopreventive agents for colon cancer (338). Treatment with the COX inhibitors increases apoptosis in the HCA-7 colon cancer cells. In these treated cells, Par-4 expression was elevated (337). This study showed that Par-4 is expressed in a colon cancer cell line. It also indicated that Par-4 is associated with apoptosis in colon cancer. However, a strong link between Par-4 and the cell death observed in these treated cells has not been established.

The viral oncogene v-Src downregulates the mRNA expression of four tumor suppressor genes. One of these tumor suppressor genes is Par-4 (319). In another study, it has been reported that v-Src reduces Par-4 protein expression (339). Both of these studies were done in fibroblasts. Nevertheless, the results of these two studies are particularly relevant in colon cancer. The cellular homologue of v-Src, c-Src, is highly implicated in colon cancer. C-Src and v-Src are members of the Src family of
nonreceptor tyrosine kinases. The protein level and kinase activity of c-Src have been observed to be increasing as colon cancer progresses into the advanced stages, including up to metastases (340). C-Src has been shown to be involved in colon cancer tumorigenesis and in metastasis. It is possible that one of the mechanisms by which c-Src promotes colon cancer tumorigenesis and metastasis is by reducing the susceptibility of colon cancer cells to apoptosis. Apoptosis has been shown to be reduced during colon cancer progression (341). Metastatic colon carcinoma cells have decreased expression of pro-apoptotic proteins and increased levels of anti-apoptotic proteins (208). Previous results that show that the viral homologue of c-Src downregulates Par-4 expression point to the possibility that c-Src may also reduce the expression of a pro-apoptotic protein, Par-4 in colon cancer.

V-src was the first oncogene identified. The name is derived from “sarcoma” from which a tumor-causing virus (Rous sarcoma virus) was first isolated by Peyton Rous (342). Rous sarcoma virus contains the v-Src gene, in addition to the gag, pol, and env genes found in other viruses (343). Subsequently, it was shown that it is the v-Src that imparted to the virus the capability to induce tumor formation (344, 345). c-Src, the human homolog, has sequences closely related to v-Src, and is ubiquitously expressed in human tissues (346, 347). C-SRC is a proto-oncogene. It does not typically cause cancers because the protein, c-Src, is kept inactive.
Both v-Src and c-Src code for nonreceptor tyrosine kinases (348, 349). Cells also contain receptor tyrosine kinases. One difference between the nonreceptor and receptor tyrosine kinases (RTK's) is the presence of transmembrane domains in the receptor tyrosine kinases. RTK's span the membrane by virtue of these transmembrane domains. Examples of RTK's are the insulin receptor (350, 351) and the epidermal growth factor receptor (352). Tyrosine kinases, whether receptor or nonreceptor, phosphorylate tyrosine residues.

V-Src and c-Src are 98% similar in their amino acid sequence. A key difference between v-Src and c-Src is the presence of a tyrosine residue, Y527 in avian c-Src, or Y530 in mammalian c-Src (353) (Figure 2-1). When this tyrosine is phosphorylated by C-terminal Src kinase (Csk) (354), the phosphorylated residue interacts with the SH2 domain. This folds the protein, making its kinase domain inaccessible and inactive. In v-Src, the tyrosine phosphorylation site in the C-terminus site is absent and the C-terminus does not interact with the SH2 domain. The kinase domain is exposed and is active (355). A mutation in c-Src, Src531, has been identified in a subset of colon cancer samples. In the mutant, the codon for site 531 is changed to a stop codon. The protein is truncated immediately following the tyrosine phosphorylation site (which is Tyrosine 530 in the human sequence). In the mutant Src 531 protein, the kinase domain is exposed and Src531 is constitutively active (356).
Figure 2-1. **Protein domain organization of c-Src, Src531, and v-Src.** The c-Src, and v-Src sequences are very similar except for the absence of the Y530 residue in the C-terminus of v-Src. C-Src folds back into itself, covering its kinase domain, as phosphorylated Y530 interacts with the SH2 domain. Although phosphorylated Y530 is necessary for folding, it is not sufficient. Src531 also has phosphorylated Y530 but is active. It is supposed that the C-terminal residues truncated in Src531 are also necessary for folding. C-Src, Src531, and v-Src are capable of autophosphorylation. The site of this autophosphorylation is Y418. The domains indicated are SH4 (4 in the figure). SH4 contains a myristoylation site that enables c-Src/v-Src to localize to the membrane. The SH3 domain is involved in protein-protein interactions. The SH2 domain mediates interaction with phosphorylated tyrosine residues.
Par-4 is downregulated in a number of cancers. Par-4 is decreased in renal cell carcinoma cells (258), cholangiocarcinomas (259), prostate carcinomas (311), lung cancers (262), in endometrial cancer cells and tissue (261, 320), and in different central nervous system tumor cells (357). In a number of these cell types, Par-4 overexpression has been tested and proven effective in causing apoptosis by itself (320, 357) or in combination with apoptotic agents (258, 320). This leads us to hypothesize that Par-4 may be downregulated by c-Src in colon cancer, and that overexpression of Par-4 in colon cancer can increase cell death by itself or in combination with another apoptotic stimulus.

In this study, the apoptotic stimulus that is combined with Par-4 overexpression is 5-fluorouracil (5-FU). 5-FU is the foundation of most current colon cancer chemotherapeutic regimens (214). As has been discussed in Chapter 1, 5-FU treatment can result in inhibition of DNA and RNA synthesis, and misincorporation of 5-FU and uracil in the DNA. Colon cancer cells and tissues undergo apoptosis in response to 5-FU treatment. In the clinic, it has been shown that continuous infusion of 5-FU given before surgery significantly increases apoptosis in the colon tumor (358). Both intrinsic and extrinsic pathways of apoptosis are involved in response to 5-FU-treatment, as evidenced by the activation of caspase-9 (359), caspase-8 and caspase-3 (360).
DNA damage occurs as 5-FU is misincorporated into the DNA (361). This can also occur as dUTP is used by DNA polymerase, as a result of dUTP accumulation. The cellular pool of dUTP increases as thymidylate synthase is inhibited by 5-FU (42, 43, 45, 46). DNA repair pathways are activated and the cell cycle is arrested as the misincorporated 5-FU and uracil is removed from the DNA. However, as the DNA repair pathway repeatedly removes the misincorporated bases in a futile cycling process, the p53 tumor suppressor is activated. A number of the genes that p53 transcriptionally upregulates encode pro-apoptotic proteins, namely the death receptors Fas and death receptor 5, and Bcl-2 protein family members Bax, and p53 upregulated modulator of apoptosis (PUMA). The expression of these proteins promotes apoptosis activation (362).

The previous results that have been discussed lead to the possibility that Par-4 is downregulated in colon cancer, in part due to c-Src. In determining the impact of Par-4 overexpression in colon cancer, I hypothesize that overexpressing Par-4 in colon cancer can cause cell death by itself or in combination with the chemotherapeutic and apoptotic agent 5-fluorouracil. To pursue this hypothesis, Par-4 was overexpressed in a colon carcinoma cell line HT29 that has high c-Src kinase activity. Wild type (WT) and Par-4 overexpressing HT29 cells were treated with 5-FU and apoptosis was assessed. To increase the clinical applicability of the findings, in vivo experiments were also performed with the Par-4-overexpressing HT29 cells.
2.2 Materials and Methods

2.2.1 Cell Culture and patient samples
The human colon carcinoma cell lines HT29, HCT116, and SW480 were obtained from American Tissue Culture Collection and cultured in RPMI culture medium (Cellgro, Manassas, VA) containing 10% fetal bovine serum (HyClone, Logan, UT) and 1% penicillin/ streptomycin at 37°C and 5% CO₂. De-identified normal colon and matched colon cancer tissue samples were obtained from the Penn State Hershey Cancer Institute Tissue Bank.

2.2.2 Stable transfections
To overexpress active c-Src in colon carcinoma cells, SW480 cells were transfected with either empty plasmid vector pcDNA3.1 (Invitrogen, Carlsbad, CA) or pcDNA-Src 531 using Fugene 6 transfection agent (Roche Applied Science, Indianapolis, IN) according to manufacturer’s instructions. Transfectants were selected with G418 antibiotic (Gibco, Carlsbad, CA) and colonies expanded and assayed for c-Src expression and Src kinase activity. C-Src kinase expression and activity were assessed by performing Western blot analyses with antibodies for c-Src and phospho-Tyr418-Src, respectively. Tyrosine 418 is the autophosphorylation site in c-Src, which is phosphorylated upon Src activation.
To overexpress Par-4, HT29 cells were transfected with either rat *par-4* cDNA in pCB6+ plasmid vector (a generous gift from Dr. V. Rangnekar, University of Kentucky) or with the empty vector using Fugene 6 transfection agent (Roche Applied Science, Indianapolis, IN), according to manufacturer's instructions. Transfectants were selected with G418 (Gibco, Carlsbad, CA) and colonies expanded and assayed for Par-4 expression.

### 2.2.3 Western blot analyses

Cell cultures were washed with phosphate buffered saline (PBS) and the cells were lysed into lysis buffer. The buffer contains 50mM HEPES, 100 mM sodium chloride, 10 mM EDTA, 0.5 % Nonidet P-40, 10% glycerol. It is supplemented with protease and phosphatase inhibitors: 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium vanadate, 0.5 mM sodium fluoride, 5 µg/ml leupeptin, and 0.1 mM dithiothreitol. In the case of tissue samples, snap-frozen tissues were homogenized in lysis buffer using a Fisher Scientific PowerGen homogenizer (Fisher Scientific, Pittsburgh, PA). Proteins from cell lysates and tissue homogenates were quantified according to the Bradford Assay (Biorad, Hercules, CA), and 50 µg protein samples were run on sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred (Bio-Rad, Hercules, CA) to nitrocellulose or PVDF membranes, and probed for the proteins of interest. Primary antibodies that were used were rabbit anti-Par 4 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-PARP, rabbit anti-caspase-8, mouse anti-caspase-9 (Cell Signaling, Danvers, MA), rabbit anti-phosphoSrc (Y416) (Invitrogen Life Science, Carlsbad, CA), mouse anti-phosphotyrosine, mouse anti-Src (Upstate Millipore,
Billerica, MA), and mouse anti-actin (Sigma, St. Louis, MO). The appropriate secondary HRP-conjugated antibodies were used (Amersham Biosciences, Piscataway, NJ). Blots were washed and developed using the ECL Plus chemiluminescent kit (Amersham Biosciences, Piscataway, NJ).

2.2.4 Src kinase activity inhibition

Cells were plated in 6-well culture plates at $4 \times 10^5$ cells/well. After 24 hours, the culture medium was changed to medium containing 0.05% dimethylsulfoxide (vehicle) or 10 µM Src kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine (PP2).

2.2.5 Viability assay

Wild type (WT) and *par*-4-transfected (Par-4) HT 29 cells were plated in 96-well plates and incubated overnight. The culture medium was replaced with fresh medium with or without 100 µM 5-FU (Sigma-Aldrich Co., St. Louis, MO). After an additional twenty-four hours of incubation, the cell viability was assessed with the CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega Corp., Madison, WI) according to manufacturer's instructions.
2.2.6 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and Caspase-3 activity assays

Wild type and par-4 transfected cells were cultured in 6-well plates. Half of the wells were treated with 100 μM 5-FU for 24 hrs. Untreated and treated cells were harvested and assayed for apoptotic cells using the APO-DIRECT TUNEL Assay kit (BD Biosciences Pharmingen, Franklin Lakes, NJ). The cells were analyzed by flow cytometry using the BD FACScan system (BD Biosciences Pharmingen, Franklin Lakes, NJ).

Parallel experiments were set-up for caspase-3 activity assays. Cells were harvested in lysis buffer (130 mM NaCl, 1% Triton X-100, 10 mM sodium pyrophosphate). Aliquots were used for protein quantitation by the Bradford method (Biorad, Hercules, CA). Ten to 50 µl cell lysates were assayed for caspase-3 activity using the Caspase-3 Assay kit (BD Biosciences Pharmingen, Franklin Lakes, NJ). The amount of product liberated from the fluorogenic substrate in the kit was measured using a BioTek Synergy HT Multidetection Microplate Reader (BioTek US, Winooski, VT) with an excitation wavelength of 380 nm and an emission wavelength of 480 nm.

2.2.7 Cell and tissue immunofluorescence

Cells were grown in six-well culture dishes containing #1.5 glass cover slips. On the day of analysis, the culture media was removed and the cells were washed twice with PBS. In the case of tissue samples, frozen tumor tissues were cryostat sectioned by the Penn State College of Medicine Histology laboratory. Tissue sections were washed
gently twice with PBS. The cells and tissue sections were fixed with 4% paraformaldehyde for 10 min, and permeabilized using 0.1% Triton-X-100. After blocking with 10% bovine serum albumin for an hour, samples were incubated with primary antibody (1:50 dilution) in 1.5% BSA/PBS for 2 h. Primary antibodies that were used were rabbit anti-Par 4 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-cleaved caspase-3 (Cell Signaling, Danvers, MA), and rabbit anti-Ki67 (Abcam, Cambridge, MA). The samples were washed in PBS 3x and incubated in the dark with phycoerythrin-labelled anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or Cy3-labelled anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for an hour. After washing in PBS, cells were incubated in 1 μg/ml of the nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Carlsbad, CA) for 2 min. Coverslips were mounted in 95% glycerol/PBS. Images were collected using a Leica RCS SP2 AOBS confocal microscope with x63 oil immersion optics.

2.2.8 Nude mouse experiments

Two sets of nude mouse experiments were performed, with each set being done twice. Nude mice were injected subcutaneously in their flanks with 100 μl cell suspension (1 x 10^7 cells) in PBS of wild type or par-4 transfected HT29 cells. Mice were then injected intraperitoneally with either 100 μl vehicle control (PBS) or 100 μl 7.5 mg/ml 5-FU solution (in PBS) for a dosage of 30 mg 5-FU/kg mouse body weight. In the first set of experiments, 5-FU treatments were done 5, 7, 12 and 14 days after cell inoculation. Three weeks after the introduction of the cells to the mice, tumors formed were excised.
and weighed. In the second set of experiments, nude mice were injected with WT cells or with both WT and Par-4 cells. In the groups that received both WT and Par-4 cells, the two different cell types were injected in opposite flanks. The advantage of this strategy is to be able to compare the growth of the WT and Par-4 tumors in the same organism. It ensures that the two different cell types are exposed to the same amount of 5-FU. Moreover, it enables the observation of any effect of Par-4 overexpressing cells on WT cells. 5-FU treatments were done 5, 7, 12, 14, 21, and 28 days after cell inoculation. The growth of the tumors was monitored weekly by measuring the length and width of the tumors. Tumor volume was calculated = length x length x width x 0.5. When tumors have exceeded 2 cm in length or width, mice were sacrificed and tumors were excised. Tumor samples from all nude mouse experiments were flash frozen in liquid nitrogen and stored at -80°C for subsequent analyses.

2.2.9 TUNEL assay in tissue sections

The TUNEL assay was performed on mouse tumor tissues to detect internucleosomal DNA cleavage that occurs in apoptosis. The In Situ Cell Death Detection Kit, Fluorescein was used (Roche Applied Science, Mannheim, Germany). Sections were fixed in freshly prepared 4% paraformaldehyde in PBS, pH 7.4 for 20 minutes. The formaldehyde was replaced with PBS and the slides were left in the PBS for 0 minutes. The slides were permeabilized in freshly prepared 0.1% Triton X-100, 0.1% sodium citrate solution, for 2 minutes on ice. The TUNEL assay is based on the observation that DNA is cleaved in apoptosis, resulting in double-stranded and single-stranded breaks.
In the assay, a terminal deoxynucleotidyl transferase (TdT) enzyme transfers labeled deoxyuridine triphosphate (dUTP) to these DNA breaks in a template-independent polymerization reaction. As a positive control, two tissue sections were treated with a DNA endonuclease, TACS-nuclease™ (Trevigen Inc., Gaithersburg, MD), for 10 minutes to induce DNA strand breaks. The TUNEL reaction mixture containing the fluorescein-labelled dUTP’s and TdT was added to the sample and to the positive control slides. As a negative control, a section was incubated with the fluorescein-labelled dUTP’s but without the TdT. Slides were incubated with the TUNEL reaction mix for 1 hour in a humidified chamber in a 37°C incubator. The sections were rinsed 3x with PBS. Nuclei were stained with DAPI and mounting was done with 95%glycerol/PBS. Images were acquired using the Leica RCS SP2 AOBS confocal microscope with x63 oil immersion optics (Leica Microsystems Inc., Bannockburn, IL).

2.3 Results

2.3.1 Colon cancer tissue and cells express Par-4

Par-4 is downregulated in a number of cancers. Given that v-Src inhibits Par-4 expression and the cellular homologue of v-Src, c-Src, is implicated in colon cancer, there is a possibility that Par-4 is downregulated in colon cancer. Endogenous expression of Par-4 was examined in matched normal and cancerous colon tissue, and in colon cancer cell lines. Par-4 is expressed in normal colon and colon cancer tissue,
albeit downregulated in some samples (Figure 2-2A). Colon cancer cell lines also endogenously express Par-4 protein (Figure 2-2B).

2.3.2 Overexpression of c-Src does not downregulate Par-4 protein expression in colon cancer.

The colon cancer tissue samples came from patients that had different stages of disease. There are other factors that can regulate Par-4 levels, e.g. exposure to other apoptotic stimuli, expression of oncogenic Ras. Thus, to more effectively study the impact of c-Src on Par-4 expression in colon cancer cells, the constitutively active c-Src mutant, Src531, was overexpressed in the colon cancer cell line SW480. To ensure that any changes in Par-4 levels resulting from Src overexpression are not a result of the plasmid vector, SW480 cells were also stably transfected with the empty plasmid pcDNA3.1 (mock). The mock-transfected cells and two Src531-transfected clones were compared for Par-4 and c-Src expression. The two Src531 clones that were used had variations in c-Src levels and activity. Clone 3 had significantly higher levels of c-Src than the mock-transfected cells. On the other hand, Src 531 clone 4 did not appear to have much higher c-Src levels. Nevertheless, both clones displayed high Src kinase activity, as indicated by higher levels of phosphorylated c-Src. Increased Src activity in SW480 cells did not downregulate Par-4 protein expression. (Figure 2-3).
Figure 2-2. Par-4 is endogenously expressed in normal colon and colon cancer.
A) De-identified matched normal colon (N) and colon tumor (T) tissue, and B) colon cancer cells were homogenized in lysis buffer. Equal amounts of protein were used for Western blot analyses for Par-4 and actin (as loading control).
Figure 2-3. Increasing c-Src level and activity did not alter Par-4 expression in SW480 colon cancer cells. Cell lysates from mock- and Src531-transfected SW480 clones were subjected to Western blot analyses for Par-4, c-Src, phospho-Src (at tyrosine 416 site), and actin (as loading control).

2.3.3 Inhibition of Src kinase activity does not result in upregulation of Par-4 expression in colon cancer.

The HT29 colon carcinoma cell line expresses high Src kinase activity (363) and is therefore, used in studies involving the impact of c-Src activity in colon cancer. To again examine whether c-Src activity in colon cancer cells can downregulate Par-4, HT29 cells were treated with a Src kinase inhibitor, 4-amino-5-(4-chlorophenyl)-7-
(dimethylethyl)pyrazolo[3,4-\(d\)]pyrimidine (PP2). To limit the effects of PP2 on other kinases, cells were treated with 10 \(\mu\)M PP2 for 48 H (364, 365). The PP2-treated cells were assessed for Par-4 expression (Figure 2-4A). Inhibiting Src kinase activity did not result in a decrease in Par-4 protein. To confirm that Src kinase activity was inhibited, Western blot analyses for tyrosine phosphorylated proteins were performed. Given that c-Src is a tyrosine kinase and that PP2 is a widely utilized Src kinase inhibitor, assessment of tyrosine phosphorylation is a suitable method to evaluate the effectiveness of the PP2. Nevertheless, to verify that Src kinase activity is inhibited with the PP2 treatment, Western blot analyses for phosphorylation of the autophosphorylation site of c-Src (Tyr418) was assessed. PP2 treatment resulted in decreased phospho-Src levels. C-Src expression levels were not affected (Figure 2-4B). The results of this experiment suggest that c-Src does not regulate Par-4 expression in colon cancer.

2.3.4 Par-4 overexpression increases apoptosis \textit{in vitro} in response to 5-fluorouracil (5-FU)

It has been shown that Par-4 overexpression is effective in inducing apoptosis by itself or in combination with apoptotic stimuli, even in cell types that endogenously express Par-4. Wild type (WT) HT29 colon carcinoma cells were transfected with the plasmid construct pCB6+par-4 to overexpress Par-4 protein. The plasmid construct pCB6+par-4 encodes the rat Par-4 sequence. This construct has been shown to induce apoptosis in previous studies by Dr. Rangnekar and his colleagues. Although the human and Par-
Figure 2-4. Inhibiting c-Src kinase activity with PP2 did not increase Par-4 expression. A) Cell lysates from HT29 cells that were treated with vehicle (- PP2) or the Src kinase inhibitor PP2 were analyzed for expression of tyrosine phosphorylated proteins, Par-4 and actin (as loading control). B) In a replicate of the experiment, inhibition of Src kinase activity was verified by Western blot analysis for c-Src, phospho-Src (at tyrosine 416 site), and actin (as loading control).

4 sequences are not 100% identical (Figure 1-11), it has been shown that the Par-4 domains that are relevant to Par-4 pro-apoptotic activity, the SAC domain and the leucine zipper, are identical in the two sequences, with the exception that in one site, the respective amino acids in the two sequences are similar. Par-4 was overexpressed in HT29 colon carcinoma cells (Figure 2-5A). As can be seen in the figure, the
amplification of Par-4 expression in response to transfection with the Par-4 construct was evident.

Par-4 overexpression alone can induce apoptosis or it can sensitize cells to apoptotic stimuli. The possibility that Par-4 can increase apoptosis by itself or in combination with 5-FU was explored. Viability assays were performed to confirm that HT29 cells are sensitive to an effective 5-FU concentration found in literature, 100 µM 5-FU (366). Both WT and Par-4 overexpressing cells had a 50% decrease in cell viability in response to 5-FU (Figure 2-5B).

Apoptosis was assessed using more than one method. Given that the different biochemical changes that are associated with apoptosis can also be induced by other processes, necrosis and autophagy, it is recommended that more than one assay for apoptosis be performed (105). Apoptosis was assessed by using four methods: Western blot analyses for cleavage of poly (ADP-ribose) polymerase (PARP), terminal dUTP Nick-End Labeling (TUNEL) assay, caspase-3 activity assay, and nuclear staining.

Poly (ADP-ribose) polymerase (PARP) is a nuclear enzyme that is cleaved in cells undergoing apoptosis. The 116 kDa PARP protein is degraded to an 89 kDa fragment (367). No cleavage of PARP was detected in the Par-4 transfected cells. On the other hand, treating Par-4 overexpressing cells with 5-FU increased PARP cleavage (Figure 2-5C).
A

Wild type

Par-4 transfected

Par-4

Actin

B

![Bar chart showing mean fluorescence (relative to untreated WT cells) for WT and Par4 cell types with and without FU.](chart.png)
C

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<td>5-FU +</td>
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Uncleaved PARP (116kDa)
Cleaved PARP (89 kDa)

D

Cell type

- fold change in apoptotic rate (relative to untreated HT29 cells)

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Figure 2-5. Par-4 overexpression increases 5-FU mediated apoptosis. A) HT29 cells were stably transfected with the plasmid construct pcB6+par-4. Cell lysates from WT HT29 and a Par-4-overexpressing clone (the clone used for the study) were analyzed for Par-4 expression by Western blot. Actin expression was assessed as a loading control. HT29 cells and one Par-4 transfected clone were untreated or treated with 100 µM 5-FU for 24 hours. B) Cell Viability Assay. Results are given as average mean fluorescence (relative to untreated WT cells + SE. * indicate significant difference vs. untreated cells. C) PARP Cleavage analysis. Cell lysates were analyzed for the cleavage of PARP. The uncleaved 116 kDa protein and the 89 kDa cleaved fragment are shown. D) TUNEL assay of cells, as detected by flow cytometry. Results are given as average fold increase in apoptosis over untreated WT cells + SE. The experiment was repeated three times. * indicate significant difference vs. untreated cells. E) Caspase-3 activity assay. Data shown are the mean fluorescence intensities of the product formed from the caspase-3 reaction normalized against the protein concentrations of the lysates +SE. * indicate significant difference vs. untreated WT
The terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) assay is performed to detect nicked DNA ends that are generated when DNA is cleaved during apoptosis. In the APO-DIRECT™ TUNEL Assay kit, the enzyme terminal deoxynucleotidyl transferase (TdT) attaches fluorescein isothiocyanate (FITC)-labelled dUTP’s to the nicked DNA ends. The fluorescence signal is detected using flow cytometry. The signal is proportional to the amount of nicked DNA ends, indicating the extent of apoptosis in a cell sample. The fluorescence readings of the treated cells were compared to untreated WT cells (Figure 2-5D). Par-4 overexpression alone did not increase apoptosis significantly in the HT 29 colon cancer cells (p=0.14). Although WT HT29 cells were not susceptible to 5-FU at the conditions used for the study (100 µM 5-FU for 24 hours), Par-4 overexpression in combination with 5-FU significantly increased apoptosis (WT w/o 5-FU vs Par-4 with 5-FU, p =0.0086).

Increased caspase-3 activity is an indication of the activation of the extrinsic and/or intrinsic pathway of apoptosis. Caspase-3 activity was assayed using a fluorogenic substrate N-acetyl-DEVD-AMC (7-amino-4-methylcoumarin). Active caspase-3 cleaves at the site after DEVD because of caspase-3 specificity. This releases the fluorescent AMC. A fluorescent plate reader was used to measure the fluorescent signal in the different treatments. Higher fluorescence readings indicated more apoptosis resulting
from a treatment. Par-4 overexpression did not result in a significant increase in caspase-3 activity assay (p=0.35) (Figure 2-5E). On the other hand, similar to results with the TUNEL assay, combining Par-4 overexpression with 5-FU treatment resulted in a significant increase in caspase 3- activity (WT w/o 5-FU vs Par-4 with 5-FU, p =0.0016).

The morphologic changes associated with apoptosis include condensation of the nucleus and chromatin, and nuclear fragmentation. These can be visualized by staining the cells with a nuclear stain such as 4',6-diamidino-2-phenylindole (DAPI). DAPI is a fluorescent stain that binds to DNA (368). Nuclear condensation and fragmentation can be seen in Par-4 overexpressing cells (Figure 2-5F), whether they were untreated or treated with 5-FU.

The results of the different assays demonstrate that Par-4 overexpression is not sufficient to increases apoptosis. However, Par-4 overexpression in combination with 5-FU significantly increases cell death.

2.3.5 Par-4 overexpressing tumors had reduced tumor size.

Par-4 overexpression increased apoptosis in response to 5-FU in vitro. To assess the effects of Par-4 overexpression in vivo, wild type (WT) and Par-4-overexpressing HT29 cells were injected subcutaneously in nude mice. Given that Par-4 overexpression was not sufficient to induce apoptosis in vitro, the effects of Par-4 overexpression and 5-FU treatment on colon tumor growth were determined. The mice were injected
intraperitoneally, with either PBS (vehicle) or 5-FU. After three weeks, tumors were excised and weighed. Par-4 overexpressing tumors had lower weight than WT tumors, although the difference was not statistically significant based on student’s t-test. Treating these mice with Par-4 tumors with 5-FU further reduced the weight of the tumors formed (Figure 2-6A). The difference was not statistically significant.

To monitor tumor growth more closely, additional nude mice were inoculated with WT cells into the right flank. Half of these mice were also injected with Par-4 overexpressing cells in the left flank. The mice were treated with PBS or 5-FU. Tumor volumes were measured weekly. The purpose of this experimental design is myriad. First, this set-up enables the comparison of WT and Par-4 cells that are exposed to the same conditions that occur in a single mouse. Second, the WT and Par-4 cells are subjected to identical 5-FU plasma concentrations. This is critical given that an individual metabolizes 5-FU differently. Thus, even if mice are given fixed doses of 5-FU, the actual concentration of 5-FU that reaches the xenografts would vary from mouse to mouse, depending on the metabolic activity of each mouse. The experiment was done twice, with 5 mice per treatment group in each treatment group. As the tumors grew to more than 2 cm in either length or width, the mice had to be sacrificed in accordance with the rules of the Institutional Animal Care and Use Committee. Par-4 tumors grew more slowly than the WT tumors. The average tumor volume of the Par-4 tumors was significantly lower than the WT tumors volumes at 3 weeks after inoculation (p=0.017) (Figure 2-6B). On the other hand, 5-FU treatment was not effective in reducing the growth of the WT and Par-4 tumors. To ensure that valid conclusions are made, the graphs of the average tumor
Figure 2-6. Par-4 overexpression decreases tumor growth in vivo. WT and Par-4 cells were injected subcutaneously into the flanks of nude mice. Half of the mice were injected intraperitoneally with either PBS or 30 mg/kg 5-FU. A) After three weeks, tumors were excised and weighed. The experiment was repeated twice, with a total of 9-10 mice per treatment group. Data are averages of tumor weights ± SE. B) Tumor volumes were measured weekly using a caliper. The experiment was repeated twice, with a total of 10 mice per treatment group. Data points indicate the average tumor volumes ±SE. * indicate significant difference in tumor volume vs WT tumors at the particular time point.
volumes only include data from time points where there are at least three mice left in a
treatment group. Including tumor averages when there are only 2 mice left per treatment
group can skew the data as mice with the bigger tumor volumes are sacrificed. Given
this strict criteria, only data collected from 3 weeks was graphed although there were
mice that were observed up to 9 weeks after cell injection.

2.3.6 Par-4 overexpression and 5-FU treatment increases caspase activation in vivo

The decreased tumor growth of Par-4 tumors can be a result of increased apoptosis,
decreased cellular proliferation, or both. Caspase activation was assessed in WT and
Par-4 tumors. During apoptosis, the initiator caspases procaspase-8 and procaspase-9
are cleaved and activated, depending on the stimuli. There were no indications that
procaspase-9 was cleaved in the WT tumors, even with 5-FU treatment (Figure 2-7). On
the other hand, procaspase-9 was cleaved in the Par-4 tumors. There was no increase
in caspase-9 activation with 5-FU treatment in the Par-4 tumors. Procaspsase-8 was
cleaved even in the WT tumors. Treating these mice with 5-FU did not result in
increased caspase-8 activation. Par-4 overexpression alone did not result in more
procaspase-8 cleavage. However, there is an indication that combining Par-4
overexpression with 5-FU treatment induced further caspase-8 activation.
Figure 2-7. Par-4 overexpressing tumors exhibited increased procaspase-9 cleavage. WT and Par-4 tumors were homogenized in lysis buffer. Equivalent amounts of protein were subjected to Western blot analyses for caspase-8 and caspase-9. Actin was used as loading control. Densitometric analyses were performed. Percent cleavage = [(intensity of cleaved band)/(intensity of cleaved band + intensity of uncleaved band)] x 100
The end stage of apoptosis can be monitored by observing the extent of internucleosomal degradation of DNA. This degradation can be assayed using the TUNEL method. Although positive control slides showed a good signal (up to 97% of the cells in the field), most of the sample slides did not have any signal. The nuclei in these sample slides were stained well with DAPI (Figure 2-8). Tissues from three mice per treatment, (4 mice in the Par-4 overexpressing group), were analyzed, with three fields imaged from each tissue sample. Although there were a few images from the different tissues that showed that there were more than 10% TUNEL (+), indicating apoptotic nuclei, most images had no TUNEL (+) nuclei (Table 2-1). This suggests that DNA fragmentation was not prevalent in any of the samples, including in Par-4 overexpressing tumors from 5-FU treated mice.

The TUNEL assay enables the detection of cleavage of DNA that occurs in the course of apoptosis. DNA is cleaved by the caspase-activated DNase (CAD). This DNase is activated by caspase-3. In assessing apoptosis, results can depend on the particular time point a parameter is measured. Therefore, to understand the apoptotic process occurring in response to a treatment, different stages of apoptosis can be studied. After procaspases are cleaved and before DNA is cleaved in the course of apoptosis, the effector caspase-3 is activated. To assess cleavage of caspase-3 in the mouse tissues, immunofluorescence to detect cleaved caspase-3 was performed. Procaspsase-3 (35 kDa) is cleaved into catalytically active 17 and 19 kDa fragments during apoptosis.
Figure 2-8. Apoptosis was not detected by TUNEL assay in WT and Par-4 tumors, even with treatment with 5-FU. WT and Par-4 tumors were cryosectioned and the TUNEL assay was performed. Positive control slides were prepared by treating tissue sections with DNA endonuclease prior to the assay. Images were acquired sequentially with a Leica confocal microscope with x63 oil immersion optics.
Table 2-1. Most of the tissues did not exhibit apoptosis based on percentage of TUNEL (+) nuclei. Percentage of nuclei that were TUNEL (+) in different tissue sections from mice with WT or Par-4 tumors that were given the PBS vehicle or 5-FU. Tissues from 3 mice (4 mice in the Par4 group) were processed and data were collected from at least three images from one tissue section. The numbers in red indicate that there are a few images that showed TUNEL positivity.

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Thus, to assess caspase-3 activity, immunofluorescence can be used to detect the 17 and 19 kDa proteins. Cleaved caspase-3 immunofluorescence results show that Par-4 overexpressing tumors had higher % cleaved caspase-3 expression (Figure 2-9A). Par-4 overexpression did not significantly increase caspase-3 cleavage in response to 5-FU treatment (Figure 2-9B).
Figure 2-9. Par-4 overexpression increased caspase-3 cleavage in vivo. A) WT and Par-4 tumors were cryosectioned and subjected to immunofluorescence. Tissues were probed with anti-cleaved caspase-3 antibody (red). Nuclei were stained with DAPI (blue). Images were acquired sequentially with a Leica confocal microscope with x63 oil immersion optics. B) % Cleaved caspase in tissue sections = (number of cells positive for cleaved caspase-3 expression/number of DAPI-positive nuclei) x 100. The p-values on top of the bars indicate p-values from performing student’s t-test between the % cleaved caspase-3 values of the treatment vs those of the WT.
The results of the different assays performed suggest that Par-4 overexpression by itself or in combination with 5-FU can promote apoptosis by increasing caspase-8, caspase-9 and caspase-3 activation. However, combining Par-4 overexpression with 5-FU did not appear to increase internucleosomal DNA degradation.

2.3.7 Par-4 overexpression decreases proliferation in tumors

To assess cell proliferation in the WT and Par-4 tumors, tissue immunofluorescence for the Ki67 protein was performed. Ki67 is a nuclear antigen that is expressed only by proliferating cells. Par-4 overexpressing tumors had a significantly lower expression of Ki67(+) cells than (p=0.0007) than the WT tumors (Figure 2-10), indicating a lower percentage of proliferating cells. Although the Par-4 tumors from 5-FU treated mice had a seemingly higher percentage of Ki67 (+) cells than the Par-4 tumors from PBS-treated mice, the difference was not significant (p=0.23) Moreover, percentage of Ki67 (+) cells in the Par-4 tumors from 5-FU-treated mice was still less (albeit statistically insignificant) than the WT tumors (p=0.092) (Table 2-2).

2.3.8 Par-4 is in the cytoplasm in colon cancer cells.

The location of Par-4 in a cell has been shown to be correlated to the susceptibility of the cell type to Par-4-mediated apoptosis. Cells that have Par-4 localized in the cytoplasm are resistant to Par-4 induced apoptosis. Therefore, to begin to understand the mechanism behind the observation that Par-4 overexpression was not sufficient to increase apoptosis in the HT29 colon cancer cell line, immunofluorescence for Par-4
Figure 2-10. Par-4 overexpressing tumors had decreased Ki67 expression. WT and Par-4 tumors were cryosectioned and subjected to immunofluorescence. Tissues were probed with anti-Ki67 antibody. Nuclei were stained with DAPI. Images were acquired sequentially with a Leica confocal microscope with x63 oil immersion optics. Percent Ki67 (+) cells = (number of cells positive for Ki67 expression/number of DAPI-positive nuclei) x 100
Table 2-2. Par-4 overexpression resulted in inhibition of cellular proliferation in tumors. Percentage of cells that were Ki67 (+) in different tissue sections from mice with WT or Par-4 tumors that were given the PBS vehicle or 5-FU. The numbers in red indicate outlier values that were not used to calculate the mean. Outliers were identified based on median absolute deviation (http://rfd.uoregon.edu/files/rfd/StatisticalResources/outl.txt). (Appendix A)

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<td>7.9</td>
<td>17</td>
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was performed. In two colon cancer cell lines HT29 and SW480, Par-4 is detected in the cytoplasm, but not in the nuclei (Figure 2-11).

**Figure 2-11.** Par-4 is localized in the cytoplasm in colon cancer cells. HT29 and SW480 colon cancer cells were grown on cover slips. Immunocytochemical localization for Par-4 was performed with rabbit anti-Par-4 primary antibody and phycoerythrin-conjugated secondary antibody (red). Nuclei were stained with DAPI. Images were acquired sequentially with a Leica confocal microscope with x63 oil immersion optics. Images acquired in the rhodamine channel (which can be used to detect signals from phycoerythrin) and DAPI channel were merged using Leica software.
2.4 Discussion

Prostate apoptosis response protein-4 has been shown to be effective in either causing or promoting apoptosis in different cell types. In this chapter, it has been demonstrated that overexpressing Par-4 in colon cancer cells causes cell death in combination with the chemotherapeutic and apoptotic agent 5-fluorouracil.

The viral oncogene v-Src has been shown to decrease mRNA (319) and protein expression (339) of Par-4 in fibroblasts. It has been demonstrated in this dissertation work, however, that Par-4 is not downregulated by the cellular homologue of v-Src, c-Src, in colon cancer cells. Although v-Src and c-Src are 98% similar, it has also been shown previously that the effects of these two proteins on gene expression patterns are not necessarily identical. Their discordant effects are not entirely because of the inhibition of c-Src by Csk. Microarray analyses have been formed in Csk-deficient fibroblasts transformed with v-Src or with c-Src. In Csk-deficient fibroblasts, the Tyr530 domain of c-Src is not phosphorylated. Thus, foreseeably, the kinase domain is exposed (Figure 2-1). The microarray experiments showed that c-Src altered the expression of 141 genes while v-Src altered the expression of 655 genes, out of the 37290 genes analyzed (369). It is possible that Par-4 is one of the genes that v-Src regulates but c-Src does not affect. Preliminary experiments in our laboratory did not show any increase in promoter hypermethylation of the Par-4 promoter in HT29 cells or any decrease in Dnmt1 protein level. Thus, the decrease in Dnmt1 expression that was seen in the v-Src transformed fibroblasts that is implicated in Par-4 downregulated may
not be occurring in colon cancer cells that have high c-Src activity, like HT29. It is possible that although v-Src downregulates Dnmt1, c-Src may not affect Dnmt1 expression.

Microarray analyses have been performed on par-4-transfected HT29 colon cancer cells in collaboration with Dr. Norman Lee of George Washington University. The result of these can explain in part how Par-4 overexpression increases apoptosis in colon cancer cells in combination with 5-FU. Par-4 has been shown to upregulate expression of pro-apoptotic genes and downregulate expression of anti-apoptotic genes. In particular, caspase-10 mRNA is increased (315). Caspase-10 is one of the initiator caspases implicated in the extrinsic pathway of apoptosis. This indicates the possibility that Par-4 overexpression promotes activation of the extrinsic pathway in colon cancer cells in vitro. There is also evidence that Par-4 is involved in the intrinsic pathway of apoptosis in colon cancer cells. Par-4 overexpression decreases Bcl-2 protein in HT29 colon cancer cells in a post-transcriptional manner. Par-4 alters Bcl-2 expression in colon cancer cells by upregulating the microRNA that represses Bcl-2 mRNA translation, miR-34a (315). Thus, Par-4 inhibits Bcl-2 expression in multiple ways. Par-4 inhibits BCL-2 transcription by its interaction with WT1 and its inhibition of NFκB activity. It also inhibits Bcl-2 mRNA translation.

The consequences of Par-4 overexpression are cell-specific. Increased Par-4 is sufficient to cause cell death in some cancers (236, 263-265). In other cell types, Par-4 overexpression serves to promote apoptosis in response to other agents but does not
cause apoptosis by itself (258, 267, 268). In cells where Par-4 is detected in the nucleus, Par-4 overexpression has been sufficient to cause apoptosis. On the other hand, in cells where Par-4 is localized to the cytoplasm, Par-4 overexpression has to be combined with another apoptotic stimulus to increase apoptosis significantly (228). Thus, to begin to elucidate the mechanism behind resistance of HT29 colon cancer cells to Par-4-induced apoptosis, Par-4 immunocytochemical localization experiments were performed. Par-4 was localized in the cytoplasm of two colon cancer cell lines, HT29 and SW480. This could explain, at least in part, why overexpression of Par-4 was not sufficient to induce apoptosis in colon cancer cells. It remains to be established why nuclear localization of Par-4 is necessary to the pro-apoptotic activity of Par-4 in many cell types, including in colon cancer. Some of the pro-apoptotic functions of Par-4 conceivably occur in the cytoplasm, specifically, its interactions with PKCζ and Akt, which result in inhibition of NFκB activity, and its association with NFκB. It is possible that for apoptosis to ensue, the activity of Par-4 in the nucleus, e.g. its interaction with WT1 and resultant downregulation of BCL-2 transcription, is required.

Par-4 overexpression increased apoptosis of HT29 colon cancer cells when combined with the apoptotic and chemotherapeutic agent 5-FU. 5-FU has been the mainstay of colon chemotherapy regimen for decades. Thus, the result that Par-4 overexpression can increase apoptosis in response to 5-FU is significant for colon cancer therapy. The viability of WT and Par-4 overexpressing cells were affected similarly by 5-FU treatment, under the conditions used in the study (100 µM for 24 hours). On the other hand, the Par-4 cells, but not the WT cells underwent apoptosis as a result of the said
treatment. It is possible that the WT cells died via other death mechanisms, such as necrosis. HT29 cells are resistant to 5-FU-mediated apoptosis. This is in part due to a mutation in its p53 gene. The tumor suppressor p53 is mutated in more than 50% of colorectal cancers (370, 371). The acute response of cells with mutant p53 to 5-FU is not apoptosis. Instead, they undergo S-phase arrest (372, 373). In cells with wild type p53, 5-FU treatment has been shown to result in Fas upregulation. The gene for Fas has a p53-responsive enhancer element (374). In cells with mutant p53 (including HT29 cells), the DNA damage that occurs in response to 5-FU does not result in Fas upregulation (374, 375). This could explain at least in part why the WT HT29 cells did not undergo apoptosis in response to 5-FU treatment. HT29 cells can be made more susceptible to apoptosis by increasing p53 expression levels, for instance, by transducing them with an adenoviral construct for wild type p53. In these transduced cells, Fas is upregulated in response to 5-FU treatment, causing apoptosis (372). This demonstrates that 5-FU-mediated apoptosis is dependent on Fas upregulation. Par-4 overexpression has been shown to increase mobilization of Fas to the surface (264) and activation of the Fas/FADD apoptotic pathway. Thus, it is possible that by combining Par-4 overexpression with 5-FU in HT29 cells, Fas is mobilized and the extrinsic pathway of apoptosis is activated despite the mutation in p53 in HT29 cells.

Another mechanism by which Par-4 overexpression can increase 5-FU mediated apoptosis is by inhibition of the anti-apoptotic consequences of 5-FU treatment in colon cancer cells. Treatment of colon cancer cells with 5-FU results in upregulation of the
anti-apoptotic protein Bcl-2, regardless of whether the cells have wild type or mutant p53 (362). On the other hand, Par-4 has been shown to downregulate Bcl-2 in many cell types. Therefore, combining Par-4 overexpression with 5-FU treatment can possibly inhibit the 5-FU-induced upregulation of Bcl-2. Another anti-apoptotic effect of 5-FU is its induction of NFκB activity in colon cancer cells. Inhibiting NFκB activity in these 5-FU treated cells can actually increase apoptosis (376, 377). Par-4 can possibly potentiate 5-FU effects by inhibiting NFκB. Through the interaction of Par-4 with PKCζ, Akt, and NFκB itself, it has been shown that Par-4 reduces NFκB activity.

Combining Par-4 overexpression with 5-FU has been effective in increasing cell death in colon cancer cells in vitro. However, to bring us closer to adopting Par-4 as part of colon cancer therapy, the effect of Par-4 overexpression on colon cancer tumor growth in vivo needed to be examined. The tumors formed by Par-4 overexpressing cells grew more slowly than the tumors formed by WT cells. 5-FU treatment, however, did not result in a further decrease in the Par-4 tumors. The reduction in tumor growth with Par-4 overexpression can be brought about by increased apoptosis or decreased cell proliferation in the tumors.

Apoptosis was assayed by assessing the activities of the initiator caspases, pro-caspase-8 and pro-caspase-9 in the tumor tissues. Western blot analyses for the uncleaved (i.e. inactive) and cleaved (active) procaspases were performed.
Procaspsase-9 was cleaved in Par-4 tumors but not in the WT tumors. 5-FU treatment did not increase caspase-9 activity. On the other hand, procaspsase-8 cleavage was not increased with Par-4 overexpression. However, combining Par-4 overexpression with 5-FU treatment increased caspase-8 activation. Par-4 overexpression increased caspase-3 cleavage. It can be surmised that this activation of caspase-3 is mediated by caspase-9, which is cleaved with Par-4 overexpression. One of the end results of caspase-3 activity, internucleosomal DNA degradation, did not appear to increase. This DNA degradation was assayed using the TUNEL method. Thus, Par-4 overexpression and 5-FU treatment may promote apoptosis in vivo via activation of the initiator caspases, caspase-8 and caspase-9.

Caspase-9 was activated in the Par-4 overexpressing tumors. It has been shown that cells within a xenograft can experience lower oxygen levels (378, 379). HT29 cells become conditioned to hypoxic (low oxygen) conditions and become more resistant to apoptosis (380) partly because of increased Bcl-2 expression (381-383). It is possible that since the tumors in the studies reported in this dissertation work were collected when they have grown to >2 cm in length or width, the tumors may be comprised more of hypoxia-resistant cells. It has been demonstrated that hypoxic areas in tumors increase as tumor volume increases (384). Hypoxia results in procaspsase-9 cleavage. This cleavage is inhibited by Bcl-2 (154, 385-387), as has been observed in hypoxia-resistant cells (380-383). In this dissertation work, it is possible that the cells in the mouse tumors experienced hypoxic conditions. In the WT tumors, Bcl-2 could have been upregulated. However, because of the ability of Par-4 to inhibit Bcl-2 expression,
Bcl-2 levels were kept low. Therefore, caspase-9 cleavage ensued. Although the mechanism just described can explain the higher procaspase-9 cleavage in the Par-4 tumors, it cannot account for the reduction in caspase-9 cleavage that occurred when mice bearing Par-4 tumors were exposed to 5-FU.

It is also possible that the downregulation of Bcl-2 as a result of Par-4 overexpression in the tumors is sufficient to result in caspase-9 activation. It has been demonstrated that transfecting Par-4 overexpressing HT29 cells with the antagomir for the miRNA regulating Bcl-2 mRNA translation, miR24a, reduces Par-4 induced apoptosis (315). This suggests that Par-4 causes apoptosis in part by reducing Bcl-2 mRNA translation and Bcl-2 protein expression.

Caspase-8 was activated in both WT and Par-4 tumors. Combining Par-4 overexpression with 5-FU treatment resulted in increased procaspase-8 cleavage. This *in vivo* finding supports the proposed explanation for the increased apoptosis observed *in vitro* with the Par-4-5-FU combination. One of the mechanisms that may explain the induction of 5-FU-mediated apoptosis by Par-4 *in vitro* is the increased mobilization of Fas by Par-4. It is highly possible that this is also occurring *in vivo*. This increased Fas surface expression mediated by Par-4 promotes the activation of the extrinsic pathway of apoptosis and the 5-FU-mediated induction of caspase-8 cleavage.
The results from the *in vitro* and *in vivo* experiments were not identical. In the *in vitro* studies, caspase-3 activity and DNA degradation were significantly increased in response to the combination of Par-4 overexpression and 5-FU treatment. On the other hand, in the *in vivo* experiments, DNA degradation was not increased with Par-4 overexpression and 5-FU treatment. Par-4 overexpression increased caspase-3 activity. However, combining Par-4 overexpression with 5-FU did not significantly increase caspase-3 cleavage in comparison with WT tumors treated with 5-FU. The challenge with studying the apoptotic process, especially by imaging, is that the time when the tissues were harvested can affect the results. Because different cells in a tissue would be at different stages of apoptosis, it is possible that by looking at one microscope field from a 10 μM section of a tissue, no cells would be seen to be undergoing the end stage of apoptosis. This could explain the variation in the data on the percentage of TUNEL positivity in the different treatments. Nevertheless, the increase in the activity of caspase-8 and caspase-9 as assessed by Western blot, indicate that apoptosis is promoted by Par-4 overexpression and 5-FU treatment. To increase overall apoptosis levels, however, especially in connection to therapy, optimization needs to be done. One possible direction is studying how different schedules and modes of administration of 5-FU can synergize with Par-4 overexpression in increasing apoptosis.

Decreased tumor growth *in vivo* can not only be brought about by increased cell death but also by an inhibition of cellular proliferation within the tumors. Proliferation was assessed in the tumor tissues by performing immunofluorescence for the presence of the Ki67 antigen in the nuclei of cells. The Ki67 protein can only be detected in the
nuclei of proliferating cells. Thus, its absence indicates that the cells are in the resting or G(0) stage (388, 389). Given this pattern of expression, Ki67 staining has been performed to detect cells that are proliferating and are non-proliferating in a tissue. In this study, Par-4 overexpression significantly decreased cellular proliferation in the tumors. This decrease in cell proliferation could be the mechanism, at least in part, behind the slower tumor growth. The increase in cell proliferation that was seen in the tumors from 5-FU treated mice is surprising, given the established inhibitory effect of 5-FU on cell proliferation.

The lack of effect on tumor growth and caspase-9 cleavage, and the increase in proliferation of the 5-FU-treated tumors are unexpected. These aberrant results may be an indication that the 5-FU treatment regimen used in the study needs to be optimized. Mice were given 30mg/kg body weight generally two times a week for the first two weeks and weekly for up to 5 weeks. It has been proposed that 5-FU be administered to nude mice in 20-50 mg/kg injections, with a total dose per week of about 100 mg/kg. The amount of 5-FU administered in the mice in the work presented in this dissertation is below the 100 mg/kg. Thus, in future studies, the effects of combining Par-4 overexpression with higher concentrations of 5-FU (i.e. close to 100 mg/kg) would need to be studied. Nevertheless, the present study still demonstrated the benefit of increased Par-4 levels in inhibiting colon cancer tumor growth in vivo.
The decreased proliferation observed in Par-4 tumors in the in vivo studies was not foreseen. The finding that Par-4 decreases cell proliferation of colon cancer cells is important given that the involvement of Par-4 in regulating cell proliferation has been studied less extensively than the role of Par-4 in apoptosis. In the case of lymphocytes, proliferation is enhanced when Par-4 levels are less than basal levels (390). In cholangiocarcinoma cells, inducing Par-4 expression with pharmacological agents results in an inhibition of proliferation (259). The mechanisms behind the inhibition of proliferation in cancer cells by Par-4 have not been established. One mechanism by which proliferation can be decreased is by slowing down cell cycle progression. In the immortalized epithelial cell line BPH-1, Par-4 overexpression has been shown to cause S-phase arrest. It has been proposed that this S-phase arrest is a result of the interaction of Par-4 with DNA topoisomerase I (Top1). Par-4 keeps Top1 from interacting with DNA, interfering with Top1 function in DNA replication and S-phase progression (241). The association of Par-4 with Top1 is particularly relevant in colon cancer cells given that, among different cancer cell lines, colon cancer (including HT29) and leukemia cell lines had generally the highest levels of Top1 (391). It is possible that colon cancer cells, with their high Top1 expression are more susceptible to Par-4-mediated inhibition of Top1 activity. Thus, colon cancer cells may respond to Par-4 overexpression with a decrease in proliferation.

In this chapter, it has been shown that overexpressing Par-4 in colon cancer cells can cause cell death in combination with the chemotherapeutic and apoptotic agent 5-fluorouracil. In vivo experiments showed that Par-4 overexpression resulted in
slower tumor growth and increased activation of caspase-9. Par-4 affects not only apoptosis but also proliferation in colon cancer cells. Par-4 overexpression inhibits cell proliferation of colon cancer cells. These observations demonstrate the potential of Par-4 overexpression to inhibit colon cancer tumor growth. In this chapter, it has also been shown that colon cancer cells express Par-4 endogenously. Thus, it is possible that endogenous Par-4 can be activated and its pro-apoptotic function be utilized to induce cell death in colon cancers. Efforts to explore this research question are described in the next chapter.
Chapter 3

Par-4 can be activated in colon cancer by treatment with Src inhibitor and 5-FU

3.1 Introduction

In Chapter 2, it has been demonstrated that Par-4 overexpression in combination with 5-FU causes cell death in colon cancer cells. It was also shown that Par-4 can be endogenously expressed in colon cancer. Given that colon cancer cells express Par-4, it is possible that endogenous Par-4 can be activated and its pro-apoptotic function be utilized. In this chapter, this possibility is explored. The specific aim of the work presented in this chapter is to elucidate how endogenous Par-4 can be activated in colon cancer, resulting in cell death.

The pro-apoptotic activity of Par-4 has been shown to be regulated by two mechanisms: posttranslational modification and subcellular localization. These two mechanisms are not independent of each other. The posttranslational modification of a protein plays a role on its location in the cell. On the other hand, it is conceivable that the location of a protein inside a cell helps determine the protein's availability to modifying enzymes, like kinases. As has been discussed, the pro-apoptotic activity of Par-4 is correlated with its subcellular localization. Cell types that express Par-4 exclusively in the cytoplasm are not susceptible to Par-4 induced apoptosis. For Par-4 to be pro-apoptotic, another
apoptotic stimulus has to be applied. Cell types that have Par-4 in the nucleus undergo
cell death in response to Par-4 overexpression. Recent studies have shown that certain
agents that cause apoptosis, such as TRAIL (334) and small molecule inhibitors of Bcl-2
(392), can induce mobilization of Par-4 to the nucleus.

Par-4 is phosphorylated by two different kinases, protein kinase A and protein kinase B
(also known as Akt). Protein kinase A phosphorylates Par-4 at threonine 163. This
phosphorylation is necessary for Par-4 mediated apoptosis. Thus, cell types, like normal
cells, that do not have high protein kinase A activity are not susceptible to Par-4 induced
apoptosis. The pro-apoptotic activity of Par-4 has been upregulated by either
overexpressing the catalytic domain of PKA or by treatment with an analog of the PKA
activator, cyclic AMP (263). Although the necessity of PKA-mediated phosphorylation of
Par-4 to the pro-apoptotic function of Par-4 has been established, the mechanism
behind how this phosphorylation actually promotes Par-4-induced apoptosis is not
known.

Par-4 is expressed endogenously by other cells in addition to colon cancer. Numerous
cell types, normal and cancerous, express Par-4. In addition, in a number of these cells,
it has been shown that Par-4 is phosphorylated at threonine 163. Thus, Par-4 is
potentially active. The question is why these cells are not undergoing cell death. This
indicates that endogenous Par-4 is kept inactive in these cells. One mechanism of
inactivation that has been elucidated is the phosphorylation of Par-4 by Akt. This phosphorylation is necessary for the binding of Par-4 to two members of the 14-3-3 protein family, 14-3-3ζ and 14-3-3η. It is proposed that the interaction of Par-4 with 14-3-3 members sequesters Par-4 in the cytoplasm, resulting in inhibition of mobilization of Par-4 to the nucleus and Par-induced apoptosis.

The 14-3-3 proteins bind to motifs containing phosphoserine (393, 394)/phosphothreonines. Seven family members have been identified, namely, β, ε, γ, η, σ, τ, and ζ. The 14-3-3 family members (which will be referred to as 14-3-3) bind to two phosphorylation motifs -- motif 1: RSX(pS)XP and motif 2: RXXX(pS)XP. The possibility that phosphorylated Par-4 binds to 14-3-3 was considered given previous observations that 14-3-3 binds to ligands that have been phosphorylated by Akt. The interaction of these substrates with 14-3-3 results in the cytoplasmic localization of these Akt substrates. For example, Bad, a pro-apoptotic Bcl-2 family member is phosphorylated by Akt at Ser99 (395). Bad is basally located in the outer mitochondrial membrane where it interacts with the anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-xL (396). In response to the activation of the PI3K/Akt pathway, Bad is phosphorylated at Ser99. This phosphorylation results in its interaction with 14-3-3 (397). The sequestration of Bad by 14-3-3 frees up Bcl-2 and Bcl-xL to interact with Bax and inhibit the activation of the intrinsic pathway of apoptosis (398). Another possibility that has been proposed is that 14-3-3 actually blocks the binding site of Bcl-xL on Bad, effectively releasing Bad from Bcl-xL. Another Akt substrate that interacts with 14-3-3 is
the Forkhead transcription factor FKHRL1/FOXO3A. FKHRL1/FOXO3A is phosphorylated by Akt at two sites, Thr32 and Ser253. Phosphorylation at both sites is required for the interaction of FKHRL1/FOXO3A with 14-3-3, specifically with 14-3-3ζ. The association of FKHRL1/FOXO3A with 14-3-3ζ keeps FKHRL1/FOXO3A from moving to the nucleus. Given that FKHRL1/FOXO3A activates the transcription of Fas ligand (306), the Akt-mediated phosphorylation of FKHRL1/FOXO3A inhibits activation of the extrinsic pathway of apoptosis. Thus, through these two substrates, Akt and 14-3-3 function to inhibit both the intrinsic and extrinsic pathways of apoptosis.

Given the cooperation of Akt and 14-3-3 in inhibiting apoptosis, it has seemed highly possible that the Akt phosphorylation of Par-4 results in an involvement of 14-3-3 in regulating Par-4 activity. It has been demonstrated that the Akt phosphorylation of Par-4 at a serine residue is necessary for interaction with 14-3-3. It has been proposed that this association with 14-3-3 inhibits movement of Par-4 to the nucleus (236). This, however, needs further investigation. Alternatively, it is possible that the interaction with 14-3-3 affects the association of Par-4 with its other partners, e.g. PKCζ, NFκB.

Akt is a serine/threonine kinase that is a downstream effector of another kinase, phosphoinositide 3-kinase/ PI 3-kinase (PI3K) (Figure 3-1). PI3K phosphorylates the D-3 position of the inositol ring of phosphatidylinositol (PI), a lipid found in membranes. This generates phosphatidylinositol-3-phosphate (PI3P) (399). Other products of PI3K
are PI3,4-bisphosphate, PI3,4,5-triphosphate (400) and PI3,5-bisphosphate. The PI3K proteins have been divided into three classes based on the stimuli that activate the PI3K, their substrate specificity, and the products of the PI3K activity. Class I PI3K phosphorylate PI4,5-bisphosphate, forming PI3,4,5-triphosphate (401). This class is divided further into class I_A and class I_B enzymes based on the proteins that activate them. Class I_A enzymes mainly act downstream of tyrosine kinases (402, 403) while Class I_B enzymes respond to the βγ-subunit of G proteins (404, 405). The Class I PI3K proteins are basally located in the cytoplasm. On the other hand, Class II PI3K's are localized to the membranes (406). They phosphorylate phosphatidylinositol and phosphatidylinositol-4-phosphate to form PI3P and PI3,4-bisphosphate, respectively (407, 408). Class III PI3K also phosphorylates phosphatidylinositol to PI3P (409). In contrast to the other PI3K classes, the class III PI3K's do not appear to be activated by external stimuli. Thus, they generate a constant level of PI3P.

The lipid products of PI3K are involved in the downstream effects of PI3K. The two lipid products of PI3K activity, PI3,4-bisphosphate and PI3,4,5-triphosphate recruit Akt to the membrane via interactions with the pleckstrin homology domain of Akt (410) (Figure 3-1). These phosphoinositides also interact with another kinase with a pleckstrin homology domain, phosphoinositide-dependent kinase 1 (PDK1) (411). PDK1 phosphorylates Akt at Thr308 (412), which is essential for Akt activation. For Akt to be fully activated, it is phosphorylated at another site, Ser473, by a kinase that has been referred to as PDK2. The function of PDK2 has been assigned to the mammalian target
**Figure 3-1. Pro-survival/anti-apoptotic effects of PI3K/Akt pathway.** PI3K phosphorylates PI4,5-bisphosphate (PIP2), forming PI3,4,5-triphosphate (PIP3). On the other hand, PTEN dephosphorylates PIP3, forming PIP2. PIP3 recruits Akt and PDK via their pleckstrin homology domains (PH). PDK phosphorylates Akt at Thr308 and another kinase, mTORC, phosphorylates Akt at Ser473. This dual phosphorylation fully activates Akt. A few of the Akt substrates are shown: FKHRL1/FOXO3A transcription factor, Bad pro-apoptotic protein, and Par-4. The phosphorylation of these substrates by Akt results in their interaction with 14-3-3. This interaction prevents the pro-apoptotic functions of these Akt substrates. The red circles in the figure denote phosphate groups. Therefore, yield higher PIP3 levels in cells. Consequently, Akt activity is also increased.
of rapamycin complex (mTORC) (413, 414). Given that the generation of lipid products are critical to the downstream effects of the PI3K/Akt pathway, an enzyme that can cleave PI3,4,5-triphosphate (PIP₃) to PI4,5-bisphosphate can inhibit the PI3K/Akt pathway. Such an enzyme is Phosphatase and tensin homolog (PTEN). PTEN is a dual specificity phosphatase -- it can remove phosphates from phosphoserines and phosphothreonines of lipids and proteins. Its lipid phosphatase activity cleaves PI3,4-bisphosphate and PI3,4,5-triphosphate (415, 416).

The PI3K/Akt pathway is upregulated in colon cancer, as evidenced by increased expression and phosphorylation of Akt in colon cancer cells and tissues (417, 418). The constitutive activation of the pathway can occur because of a number of reasons. One is because of mutations in the gene that codes for the catalytic subunit of PI3K class IA, PIK3CA. These mutations have been found in 10-20% of colorectal carcinomas (419, 420). The mutations result in PI3K variants that have higher lipid kinase activity, and therefore, yield higher PIP₃ in cells. Consequently, Akt activity is also increased (421, 422). Upregulation of the PI3K/Akt pathway also occurs when the lipid phosphatase PTEN is downregulated. It has been found that 17-35% of colorectal cancers exhibit loss of PTEN expression (423, 424). This loss can be due to gene silencing as a result of promoter methylation (425), or due to chromosomal instability at the PTEN locus 10q23 (423, 424). PTEN gene mutations have also been detected in 13-18% of colon cancers (423, 426, 427).
The upregulation of the PI3K/pathway in colon cancer, resulting in increased activity of Akt, gives us strong reason to believe that endogenous Par-4 is inactivated in colon cancer, at least in part, by Akt. For the purpose of colon cancer therapy, however, it is not only advantageous to increase apoptosis but also to inhibit metastases. This issue is particularly pertinent in colon cancer given that a high percentage of the mortalities associated with colon cancer result from metastasis. Although a few studies suggest that Akt plays a role in metastasis, it has not been shown that it drives the metastatic process. On the other hand, Src family kinases (hereafter referred to as Src) are not only involved in colon cancer tumorigenesis but also in metastatic processes (428, 429). Src has been shown to activate the PI3K/Akt pathway. Therefore, it is possible that by inhibiting Src, Akt can be downregulated and endogenous Par-4 can be activated.

The proto-oncogene c-Src, a member of the Src family of nonreceptor tyrosine kinases, is highly implicated in colon cancer. When colon cancer tissues from patients that have different stages of the disease, including metastases were analyzed, it has been found that the protein level and kinase activity of c-Src increase with colon cancer progression (340, 430). Src activity is inversely correlated with overall survival of colon cancer patients (430). Increasing c-Src expression alone can induce carcinogenesis in vivo (431). Aside from the involvement of Src in tumor formation and growth (432), Src activity promotes colon cancer metastasis (433). One of the hallmarks of cancer is the inhibition of apoptosis (434). The contribution of Src to this evasion of apoptosis in
colon cancer is not fully elucidated. Nevertheless, there is evidence that Src has an impact on cell death in colon cancer. The expression and activity of c-Src have been shown to be correlated with the ability of a colon cancer cell line to resist detachment-induced apoptosis (anoikis) (435). Overexpressing a constitutively active mutant of c-Src is sufficient to decrease anoikis. On the other hand, inhibiting c-Src alone is not sufficient for increasing apoptosis in colon cancer cell lines (365, 435, 436). However, Src inhibition promotes cell death to a number of stimuli, namely, detachment from a surface (as in anoikis), and treatment with staurosporine (435, 436), Fas ligand and oxaliplatin. The downregulation of c-Src has been shown to increase oxaliplatin-induced apoptosis by promoting caspase-8 cleavage (365). The mechanism by which Src inhibition favors caspase-8 activation in response to oxaliplatin has not been established.

It has been suggested that inhibiting c-Src promotes apoptosis, at least in part, via the downregulation of the PI3K/Akt pathway (435, 436). Increasing Src kinase activity has been shown to activate Akt (437, 438). C-Src interacts with the proline-rich domain of Akt (439), phosphorylating it at two tyrosine residues (Tyr$^{315}$ and Tyr$^{326}$). Mutating these two residues abolishes the kinase activity of Akt. The phosphorylation of these two tyrosine residues has been shown to be important for the anti-apoptotic/pro-survival effect of Akt. Src also affects Akt activity by regulating the other members of the PI3K/Akt pathway. Src promotes the recruitment of an activating kinase of Akt, PDK1 to the membrane, by the interaction of Src with tyrosine phosphorylated residues of PDK1.
A consequence of this increased mobilization of PDK1 to the membrane is increased activation of Akt. A negative regulator of the PI3K/Akt pathway, PTEN, is also affected by Src activity. PTEN has been shown to be phosphorylated by Src at tyrosine residue/s. This phosphorylation inhibits PTEN activity, thereby increasing Akt activation (441).

It has been shown in the previous chapter of this dissertation that overexpressing Par-4 in the colon carcinoma cell line HT29 increases apoptosis in response to the chemotherapeutic agent 5-fluorouracil (5-FU). Although Par-4 is endogenously expressed in colon cancer (442), albeit at lower levels than normal tissue, with respect to mRNA expression (315), it does not cause apoptosis. This suggests that it is inactivated in these cells. Previous research has shown that Par-4 can be inactivated by phosphorylation by Akt. C-Src, a kinase that is critical to colon cancer progression, has been shown to regulate the PI3K/Akt pathway, especially Akt. Results have shown that Src inhibition promotes apoptosis, in part through downregulating Akt. Therefore, I hypothesize that the inhibition of c-Src in colon cancer will decrease Akt activity, and thereby activate Par-4 and cause cell death.
3.2 Materials and Methods

* Only materials and methods that have not been discussed in the previous chapter are described.

3.2.1 Determination of the 5-FU half maximal inhibitory concentration (IC\textsubscript{50})

Cells were plated in 96-well plates. The cells were allowed to attach for 24 hours and the medium was replaced with fresh medium containing either vehicle (DMSO) or different concentrations of 5-FU. The final volume per well was 100 µl. After twenty-four hours exposure, viability was assessed by performing the MTT assay. The protocol of the Wallert and Provost Lab of Minnesota State University was adopted, and is as follows. Twenty µl 5 mg/ml MTT solution (thiazolyl blue tetrazolium bromide in PBS) was added to the wells and the cells were incubated for 3.5 h at 37°C. The media was carefully removed and 150 µl MTT solvent (4 mM HCl, 0.1% Nonidet P-40 in isopropanol) was added. After agitating the cells in the dark on an orbital shaker for 15 minutes, the absorbance at 570 nm was read with a reference filter of 630 nm. The IC\textsubscript{50} for a cell line was defined as the 5-FU concentration that induced a 50% decrease in viability compared to cells treated with vehicle alone.

3.2.2 Src kinase activity inhibition and 5-FU treatment

Cells were plated in 6-well plates at 4 x 10\textsuperscript{5} cells/well for 48 hour treatments, and at 8 x 10\textsuperscript{5} cells/well for 72 and 96 hour treatments. After 24 hours, the medium was changed to medium containing 0.05% DMSO or 10 µM Src kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-\textit{d}]pyrimidine (PP2). After an additional 24
hours, DMSO (0.1%) or 5-FU was added to a final concentration equivalent to the IC$_{50}$ for the cell type tested. In the case of HT29, 100 µM 5-FU was added. For mock and Src-531-transfected SW480 cells, 200 µM 5-FU was used.

3.2.3 TUNEL Assay

Cells were plated in 96-well plates at 6.25 x 10$^3$ cells/well. To normalize the absorbance readings obtained from the assay, the cells in the wells left after treatment were manually counted under an inverted light microscope. The averages of the counts under two fields were calculated. Apoptosis was assessed by TUNEL assay using the HT TiterTACS™ TUNEL ELISA kit (Trevigen Inc., R&D Systems, Minneapolis, MN), according to manufacturer’s instructions.

3.2.4 Immunoprecipitation

Cell lysates (50 µg) were incubated with 1 µg primary antibody overnight at 4°C. Primary antibodies that were used for immunoprecipitation were rabbit anti-Par-4 and goat anti-14-3-3σ (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti c-Src (Upstate/Millipore, Billerica, MA). The σ isoform of 14-3-3 is preferentially expressed in epithelial cells (443). Twenty µl of Protein A/G agarose beads (Santa Cruz, Santa Cruz, CA) was added. After 2 hours, the immunoprecipitation reaction was spun at 2500 rpm for 5 min. The supernatant was collected for subsequent Western blot analyses. The immunoprecipitates were washed 3x with lysis buffer. Twenty µl 2x SDS sample buffer
(0.5 M TrisHCl [pH6.8], 4.4% SDS, 20% glycerol, 0.2% bromophenol blue, 0.2M DTT) was added and heated at 95-100°C for 5 min. The beads were pelleted and the supernatant was used in Western blot analyses.

3.2.5 Subcellular localization of Par-4.

Cells were plated in 6-well plates at 8 x 10^5 cells/well. After 24 hours, the medium was changed to that containing either 0.05% DMSO or 10 µM PP2. Twenty-four hours after medium change, vehicle (0.1%) or 5-FU was added to a final concentration of 100 µM. To ensure that enough cytoplasmic and nuclear proteins were isolated, for each experiment, one well was treated with vehicle, one well with PP2, two wells with 5-FU and two wells with both PP2 and 5-FU. Seventy-two hours after addition of 5-FU, cytoplasmic and nuclear proteins were isolated using NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific Inc., Rockford, IL) according to manufacturer's instructions. The proteins in the fractions were quantitated using the BCA Assay (Thermo Scientific Inc., Rockford, IL). Western blot analyses were done for Par-4 and TATA-binding protein (TBP). TBP was detected as a loading control for the extracts. It was also used to monitor efficient separation of cytoplasmic and nuclear fractions.
3.3 Results

3.3.1 Overexpression of c-Src inhibits Par-4 activation in colon cancer.

The pro-apoptotic activity of Par-4 is increased by its phosphorylation at Thr163 by PKA. As was mentioned in the previous chapter, the constitutively active c-Src mutant, Src531, was overexpressed in the colon cancer cell line SW480. The mock-transfected cells and two Src531-transfected clones did not differ in terms of Par-4 expression. On the other hand, when phosphorylation of Par-4 at threonine 163 was analyzed, it was observed that phospho-Par-4 levels were decreased in the Src 531-overexpressors (Figure 3-2).

3.3.2 Inhibiting c-Src kinase activity with the pharmacological inhibitor PP2 increases 5-FU-mediated apoptosis.

To begin to determine the impact of c-Src on the pro-apoptotic activity of Par-4, experiments were performed to assay apoptosis in response to Src inhibition and the apoptotic and chemotherapeutic agent 5-FU. It has been demonstrated that Par-4 overexpressing HT29 cells undergo apoptosis in response to 5-FU while WT HT29 cells are resistant to 5-FU-induced apoptosis, at least under the conditions tested. Src inhibition experiments were first performed with HT29 cells. HT29 cells have high endogenous Src levels and activity (435) and are therefore representative of colon
Figure 3-2. Src kinase activity inhibits Par-4 phosphorylation at Thr163. Western blot analyses for phosphorylated Par-4 and actin (as loading control) were performed (the last two rows). To observe phosphorylation of Par-4 at Thr163, a rabbit polyclonal antibody specific for phosphorylation at the site (Cell Signaling Technology, Danvers, MA) was used. The results for Par-4, c-Src, and phospho-Src have been reported and discussed in the previous chapter (Figure 2-3). However, these were included here for the purpose of clarity.
cancer tissues from advanced stages of colon cancer that have high Src expression and activity. To decrease c-Src kinase activity, cells were treated with the pharmacological Src kinase inhibitor (PP2). Western blot analyses confirm that c-Src kinase activity was inhibited when PP2 was added, as manifested by reduction in phosphorylation at the tyrosine-418 site of c-Src (Figure 3-3).

Figure 3-3. Treating HT29 cells with the Src inhibitor PP2 decreases c-Src kinase activity. HT29 cells were plated and incubated overnight. The medium was replaced with fresh medium containing either 0.05% DMSO or 10 µM PP2. After 24 hours, vehicle (DMSO) or 5-FU was added. Cell lysates were obtained after total treatment times of 48, 72, and 96 hours. Western blot analyses for phospho-Src, c-Src and actin were performed.
Apoptosis was assessed in three ways: TUNEL assay, analysis of PARP cleavage, and caspase-3 activity assay. Apoptosis as measured using TUNEL analyses was significantly increased only after 96 h of treatment with 5-FU (p=0.0008, vs. vehicle), and with both PP2 and 5-FU (p=0.015, vs. vehicle). Src inhibition was not sufficient to increase apoptosis (p=0.78, vs. vehicle). Nevertheless, inhibiting c-Src increased 5-FU mediated apoptosis. (p =0.021, 5-FU vs Both) (Figure 3-4A). After 96 hours of treatment with 5-FU alone or with the combination of 5-FU and PP2, most of PARP is cleaved (Figure 3-4B). Caspase-3 assays confirm the increase in apoptosis seen when c-Src is inhibited along with 5-FU treatment (p=<0.0001, vs. vehicle) (Figure 3-4C).

To verify that the effect of 5-FU and PP2 on apoptosis was not specific to one colon cancer cell line, three additional cell lines were treated. These were the one clone of mock- and two clones of Src531-transfected SW480 cells (hereafter referred to as SW480 mock and SW480 Src531, respectively). To determine the appropriate 5-FU concentration to be used in these cells, MTT viability assays were performed (Appendix B). From these experiments, it was concluded that the most appropriate 5-FU concentration to use was 200 µM. Treating the mock and the Src531-transfected cells with 200 µM 5-FU resulted in 50-60% decrease in viability.
### A

**Absorbance (450 nm)/cell number**

<table>
<thead>
<tr>
<th>Incubation Time (H)</th>
<th>Vehicle</th>
<th>PP2</th>
<th>5-FU</th>
<th>Both</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 H</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>72 H</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>96 H</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

### B

**Treatment time (H)**

<table>
<thead>
<tr>
<th>Treatment time (H)</th>
<th>48 H</th>
<th>72 H</th>
<th>96 H</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM PP2</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>100 mM 5-FU</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Uncleaved PARP**

- 21%
- 25%

**Cleaved PARP**

- 59%
- 58%

**Actin**

- 0%
- 2%
- 95%
- 92%

**% PARP cleavage**

<table>
<thead>
<tr>
<th>48 H</th>
<th>72 H</th>
<th>96 H</th>
</tr>
</thead>
<tbody>
<tr>
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<td>2</td>
</tr>
<tr>
<td>95</td>
<td>92</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3-4. Inhibiting c-Src with PP2 increases 5-FU-mediated apoptosis in HT29 cells. HT29 cells were treated with vehicle (0.15% DMSO), 10 µM PP2 only, 100 µM 5-FU only, or with both PP2 and 5-FU. Apoptosis was assessed after 48, 72, and 96 hours of treatment. A) TUNEL assay was performed using an ELISA-based assay. The
bars in the graph reflect absorbance readings (at 450 nm) from the assay normalized over the number of cells in the well. Data shown are mean of at least 4 biological replicates ± SE and are representative of three independent experiments. * indicate significant difference vs. vehicle control of the same time point; † indicate significant difference vs cells treated with both PP2 and 5-FU. B) Western blot analyses of PARP cleavage was performed. Actin was used as loading control. Densitometric analyses of the uncleaved and cleaved PARP bands were performed. Percent PARP cleavage was calculated: % cleavage = [(cleaved PARP)/(uncleaved PARP + cleaved PARP)] x 100. C) Caspase-3 activity was assessed using an ELISA-based assay after 96 hours of treatment. Total protein of cell lysates was quantitated using the BCA assay. The bars in the graph reflect fluorescence readings from the assay normalized over the amount of cell lysate used. Data shown are mean ± SE from at least three independent experiments. * indicate significant difference vs. vehicle control of the same time point; † indicate significant difference vs cells treated with both PP2 and 5-FU; p=0.054 refer to the difference between cells treated with 5-FU alone and cells treated with both PP2 and 5-FU by student's t-test. D) Mock- and Src531-transfected SW480 cells were treated for 96 hours and apoptosis was assessed using the TUNEL assay. The 5-FU concentration used for SW480 mock and SW480Src531 was 200 µM based on preliminary IC50 experiments. The data shown was acquired, analyzed and presented similarly as in a). * indicate significant difference vs. vehicle control of the same time point; † indicate significant difference vs cells treated with both PP2 and 5-FU by student's t-test.

Src inhibition increased apoptosis in the two Src531-transfected clones but not in the mock-transfected clone. The three cell types were susceptible to 5-FU-induced apoptosis. PP2 treatment augmented 5-FU induced apoptosis in one of the Src531-overexpressing clones (Figure 3-4D).
3.3.3 Src kinase activity inhibition and 5-FU treatment reduce the interaction of Par-4 with Akt1 and 14-3-3σ.

One mechanism by which Src can activate Par-4 is inhibition of the association of Par-4 with Akt. Therefore, immunoprecipitation analyses were performed to assess whether Src inhibition by PP2 perturbs the Par-4/Akt interaction. Treating the cells with PP2 resulted in a decreased association of Par-4 with Akt1 (Figure 3-5A). Cells treated with 5-FU or with both PP2 and 5-FU had further reduced Par-4 interaction with Akt1. The interaction of Par-4 with Akt was significantly lower in PP2-, 5-FU, and combination-treated cells than in the vehicle-treated cells (Figure 3-5B).

Par-4 associates with 14-3-3 in a manner that is dependent on the Akt-mediated phosphorylation of Par-4. It has been proposed that the interaction of Par-4 with 14-3-3 inhibits the localization of Par-4 to the nucleus and the pro-apoptotic activity of Par-4. To determine whether Src inhibition affects Par-4/14-3-3 interaction, immunoprecipitation analyses were also performed. The association of Par-4 with the σ isoform of 14-3-3 was studied given that 14-3-3σ is the predominant isoform expressed in epithelial cells (443, 444) like colon cancer cells. Par-4 was co-immunoprecipitated with 14-3-3σ in vehicle-treated cells (Figure 3-5C). The association of Par-4 with 14-3-3σ was reduced in cells treated with PP2, 5-FU, or the combination of both. To verify that the reduction in Akt1 and 14-3-3σ that were co-immunoprecipitated with Par-4 was not a result of decreased Akt1 and 14-3-3σ expression, Western blot analyses were performed.
A

10 µM PP2  -  +  -  +
100 µM 5-FU  -  -  +  +

IP: Par-4  WB: Akt1
Par-4
10 µM PP2      - +     - +     
100 µM 5-FU   - - +     ... Par-4 (versus 
vehicle-treated cells)

Treatment
*                          *                        *

B

Fold change in immunoprecipitated Akt/immunoprecipitated Par-4 (versus vehicle-treated cells)

Vehicle PP2 5FU Both

Treatment
Figure 3-5. Src kinase activity inhibition and 5-FU treatment decrease the association of Par-4 with Akt1 and 14-3-3σ. A) Par-4 was immunoprecipitated from HT29 cells treated with vehicle, PP2, 5-FU or both PP2 and 5-FU, for 96 hours. Immunoprecipitates were analyzed by Western blot for the presence of Akt1 and Par-4. B) Densitometric analyses of the Akt and Par-4 bands were performed on results collected over three independent experiments. The band intensities of the Akt1 bands
were normalized using the corresponding Par-4 bands. The bars in the graph reflect the calculated fold change of the normalized Akt bands of PP2-, 5-FU-, or combination-treated cells over vehicle-treated cells. * indicate significant (p<0.05) difference vs. vehicle control using student's t-test. C) Immunoprecipitation was done with 14-3-3 σ antibody. The immunoprecipitates were analyzed for the presence of Par-4. D) Expression levels of Par-4, Akt1 and 14-3-3 σ were assessed on the cell lysates by Western blot. Actin was used as loading control.

Western blot analyses show that Akt1 and 14-3-3σ levels were not reduced (Figure 3-5D).

3.3.4 Par-4 is mobilized to the nucleus with c-Src kinase activity inhibition and 5-FU treatment

The pro-apoptotic activity of Par-4 has been correlated with its localization in the nucleus. To examine the possibility that Src inhibition and 5-FU treatment increase apoptosis by inducing nuclear localization of Par-4, immunocytochemical localization of Par-4 was performed after 96 hours of treatment with PP2, 5-FU, and both PP2 and 5-FU. Par-4 was localized to the cytoplasm in wild-type, vehicle treated, and PP2-treated cells. However, when cells were treated with 5-FU or the PP2 and 5-FU combination, Par-4 was observed in both the cytoplasm and the nucleus (Figure 3-6A). To confirm the results of the immunocytochemical localization experiments, subcellular fractionation was performed after 96 hours of treatment. Par-4 expression in the nuclear and cytoplasmic fractions was analyzed by Western blot analysis. In vehicle treated cells, Par-4 was detected in both the cytoplasm and the nucleus. In cells treated with 5-
A

Untreated

Vehicle

PP2
Figure 3-6. Par-4 is mobilized to the nucleus in cells treated with PP2 and 5-FU. A) Immunocytochemical localization of Par-4 was performed in untreated HT29 cells, and HT29 cells treated with vehicle, PP2, 5-FU or both PP2 and 5-FU, for 96 hours. Nuclei were visualized by counterstaining with DAPI. Images were acquired using a confocal microscope. Par-4 images are shown in the left panels. The middle panels are the DAPI images and the right panels are the overlay of Par-4 and DAPI images. B) Western blot analyses of Par-4 on cytoplasmic (C) and nuclear (N) extracts from cells treated with vehicle, PP2, 5-FU or both PP2 and 5-FU. TATA-binding protein, a nuclear protein, was used to confirm efficiency of fractionation and to serve as loading control. C) Densitometric analyses of Par-4 band intensities in the cytoplasmic and nuclear fractions were performed. The bars in the graph reflect the means of the quantitation of Par-4 in a particular compartment divided by the total amount of Par-4 in both the...
cytoplasmic (C) and nuclear (N) fractions. Error bars indicate standard error. The experiment was repeated at least three times.

FU and both PP2 and 5-FU, at least 70% of the Par-4 was found in the nucleus (Figures 3-6B and C). The subcellular localization of Par-4 was performed at least three times and the Par-4 in each compartment was quantitated via densitometric analyses. In cells treated with 5-FU and with both PP2 and 5-FU, there was an unequivocal mobilization of Par-4 to the nucleus. In cells treated with PP2 alone, however, the results were not consistent across experiments (Figure 3-6C).

3.3.5 C-Src kinase activity inhibition and 5-FU treatment downregulate the PI3K/Akt pathway.

C-Src can upregulate the PI3K/Akt pathway. To look into the involvement of the PI3K/Akt pathway on the pro-apoptotic effect of c-Src inhibition, cells treated with PP2 and/or 5-FU for 96 hours were analyzed for phospho-Akt. Src inhibition did not result in a detectable decrease in Akt phosphorylation at the Ser473 site. On the other hand, treating the cells with 5-FU or with both PP2 and 5-FU reduced Akt phosphorylation (Figure 3-7A). The experiment was repeated three times and the fold change in Akt phosphorylation versus vehicle-treated cells was calculated. Treating the cells with 5-FU or with both PP2 and 5-FU significantly decreased Akt phosphorylation at the Ser473 site (Figure 3-7B).
Figure 3-7. Src kinase activity inhibition and 5-FU treatment downregulate the PI3K/Akt pathway. A) Akt phosphorylation was assessed by Western blot analyses in HT29 cells treated with vehicle, PP2, 5-FU or both PP2 and 5-FU, for 96 hours. Actin was used as loading control. B) Densitometric analyses of phosphorylated Akt and actin bands were performed on results collected over three independent experiments. Phospho-Akt intensities were normalized using actin intensities. The bars in the graph
reflect the calculated fold change of the normalized phospho-Akt values of PP2-, 5-FU-, or combination-treated cells over vehicle-treated cells. * indicate significant difference vs. vehicle control

3.3.6 PP2 and 5-FU treatment inhibit the interaction of Par-4 with c-Src

Given the consequences of c-Src inhibition on apoptosis and on the interaction of Par-4 with Akt1 and 14-3-3σ (Figures 3-5A and B), the possibility that Par-4 associates with c-Src was explored. Results show that c-Src can be immunoprecipitated with Par-4. In addition, their interaction was decreased when cells were treated with PP2. The association of Par-4 with c-Src was further decreased when cells were incubated with 5-FU or with the PP2/5-FU combination (Figure 3-8A). The decrease in immunoprecipitated c-Src in PP2-treated cells was not a result of a reduction in c-Src protein levels, as shown by Western blot analyses (Figure 3-8B). To verify that the immunoprecipitation of c-Src with Par-4 was not a result of the reported interaction of c-Src with Akt1 (439), immunoprecipitation of c-Src was performed. Akt1 was only detected in the supernatant of the immunoprecipitation procedure (Figure 3-8C), indicating that Akt1 does not interact with c-Src in the treatment conditions tested.
A

10 µM PP2 - + - +
100 µM 5-FU - - + +
IP: Par-4  WB: c- Src
Par-4

B

10 µM PP2 - + - +
100 µM 5-FU - - + +
WB: c- Src
Actin

C

10 µM PP2 - + - +
100 µM 5-FU - - + +
p s p s p s p s
IP: c-Src  WB: Akt1
c-Src
Figure 3-8. Par-4 is associated with c-Src and is phosphorylated on tyrosine residue(s). A) Par-4 was immunoprecipitated from HT29 cells treated for 96 hours with vehicle, PP2, 5-FU or both PP2 and 5-FU. Immunoprecipitates were analyzed by Western blot for the presence of c-Src. B) Expression levels of c-Src were assessed on the cell lysates by Western blot. Actin was used as loading control. C) C-Src was immunoprecipitated from HT29 cells treated for 96 hours with vehicle, PP2, 5-FU or both PP2 and 5-FU. Immunoprecipitates (p) and aliquots of the supernates (s) from the immunoprecipitations were analyzed by Western blot for the presence of Akt1. D) Par-4 was immunoprecipitated from HT29, SW480, and HCT116 cells. Immunoprecipitates (p) and aliquots of the supernates (s) from the immunoprecipitations were analyzed by Western blot for tyrosine phosphorylation. Par-4 was immunoprecipitated from E) HT29 cells treated with vehicle or PP2 for 48, 72, or 96 hours, and F) mock SW480 and SW480Src531 cells. Immunoprecipitates were analyzed by Western blot for tyrosine phosphorylation.
Seeing the effects of inhibiting c-Src on the interactions of Par-4 with Akt1 and 14-3-3σ (Figure 3-5), the possibility that Par-4 is phosphorylated at tyrosine residues was explored. Par-4 was phosphorylated at tyrosine residues in three colon cancer cell lines (HT29, SW480, and HCT116) (Figure 3-8D). However, this tyrosine phosphorylation was not reduced in response to Src inhibition (Figure 3-8E). Nevertheless, when c-Src was overexpressed, Par-4 tyrosine phosphorylation was increased (Figure 3-8F).

3.4 Discussion

Par-4 overexpression in combination with 5-FU has been shown to cause apoptosis in colon cancer. On the other hand, given that Par-4 is often endogenously expressed in colon cancer, it is possible that pro-apoptotic activity of endogenous Par-4 can be utilized. In this chapter, it has been demonstrated that the inhibition of c-Src in colon cancer in combination with 5-FU can activate endogenous Par-4 and cause cell death.

Src inhibition was not sufficient to induce apoptosis in HT29 and SW480 colon cancer cells. On the other hand, two Src-overexpressing clones of SW480 were susceptible to PP2-induced apoptosis. It has been shown that Src inhibition alone is not sufficient to induce cell death. In the case of the Src overexpressors, it is possible that as the cells have increased levels of c-Src or increased Src kinase activity, they become more
dependent on Src activity. Because of this dependence, they become more susceptible
to the effects of a Src inhibitor. This altered behavior has been referred to as oncogene
addiction or oncogene dependence. Combining PP2 with 5-FU significantly increased
apoptosis in comparison to apoptosis induced by PP2 or 5-FU alone in most of the cell
types tested. Previous studies have shown that Par-4 is activated in response to
apoptotic stimuli like Bcl-2 inhibitors and TRAIL. Thus, the observations that c-Src
inhibition and 5-FU induces apoptosis point to the possibility that Par-4 is activated by
Src inhibition and 5-FU treatment.

The pro-apoptotic activity of Par-4 has been shown to be regulated by a number of
mechanisms. Par-4 is phosphorylated at Thr163 by PKA. This phosphorylation is
necessary for Par-4-induced apoptosis. It has been shown that Src overexpression in
colon cancer inhibits phosphorylation of Par-4 at Thr163. Although there is evidence
that PKA inhibits Src kinase activity (445), there has been no report of c-Src inhibiting
PKA activity. Thus, the decreased phosphorylation of Par-4 at the Thr163 site may not
be due to Src inhibition of PKA activity. Another plausible mechanism is that Src alters
the conformation of Par-4, making it less available to phosphorylation by PKA.

Endogenous Par-4 is phosphorylated by Akt. This phosphorylation of Par-4 by Akt, in
contrast to that mediated by PKA, inhibits the pro-apoptotic activity of Par-4. Akt can
associate with Par-4 even if the putative Akt phosphorylation site is mutated. In this
dissertation work, it was shown that Src inhibition, 5-FU treatment, and the PP2/5-FU combination significantly decreased the interaction of Par-4 with Akt.

A consequence of Par-4 phosphorylation by Akt is the interaction of Par-4 with 14-3-3. It has been shown that this Par-4/14-3-3 association inhibits mobilization of Par-4 to the nucleus and cell death. Src inhibition can activate Par-4 in part by inhibiting the interaction of Par-4 with 14-3-3. The data shows that in HT29 colon cancer cells, Par-4 endogenously associates with 14-3-3, specifically with the sigma isoform. The sigma isoform is expressed in epithelial cells (444) and is mainly localized in the cytoplasm (446). 14-3-3 proteins are highly implicated in pro-survival mechanisms (447-449).

Similar to the interaction between 14-3-3 and Par-4 (236), the sigma isoform of 14-3-3 interacts with YAP, a transcription factor that promotes p73-mediated apoptosis. This interaction sequesters YAP in the cytoplasm, keeping it from activating p73 in the nucleus (448, 450). It has been proposed that the interaction of Par-4 with 14-3-3 also keeps Par-4 from moving to the nucleus (236). The localization of Par-4 to the nucleus has been correlated with its pro-apoptotic activity (228, 236). When HT29 cells were treated with PP2, 5-FU, or the combination of PP2 and 5-FU, the interaction of Par-4 with 14-3-3σ was reduced. Par-4 was localized to the cytoplasm and the nucleus in 5-FU and PP2 5-FU-treated cells.
Src inhibition and 5-FU treatment have been shown to activate Par-4 as evidenced by decreased interaction of Par-4 with Akt and 14-3-3σ, and increased mobilization to the nucleus. Although Src inhibition reduced the Par-4/Akt and Par-4/14-3-3σ interactions, it was not sufficient to consistently induce mobilization of Par-4 to the membrane. Similarly, Src inhibition with PP2 did not inhibit Akt activity significantly, as assayed by Western blot analysis for phosphorylation of Akt at the Ser473 site. This indicates that Src may be promoting the interaction of Par-4 with Akt and 14-3-3σ by a mechanism that is independent of Akt activity. A possible scenario is that Src alters the conformation of Par-4, therefore, favoring its interaction with Akt and 14-3-3σ. It has been shown that Src interacts with Par-4. Although c-Src has been shown to associate with Akt1 (439), Akt1 was not detected in c-Src immunoprecipitates under the conditions tested. This suggests that Par-4 and Src interact independently of the potential association of c-Src with Akt1. C-Src can potentially associate with Par-4 via the Src SH2 and/or SH3 domains (Figure 2-1). SH2 domains binds to phosphotyrosine-containing protein motifs (451). Although Par-4 is phosphorylated at tyrosine site(s), it does not contain any predicted Src family SH2 domain binding motifs. On the other hand, the Par-4 sequence has one putative binding site for class I SH3 domains (452), a motif which c-Src SH3 selectively binds (453). C-Src may associate with Par-4 via this binding site.
The observation that c-Src associates with Par-4 led to the question of whether Par-4 is phosphorylated by c-Src. The human Par-4 sequence has six tyrosine sites (454), with three of these sites being predicted to be phosphorylated (455). In this dissertation work, it has been shown that Par-4 is phosphorylated at tyrosine residue/s. Although inhibition of Src kinase activity reduced the interaction of Par-4 with c-Src, tyrosine phosphorylation of Par-4 was not decreased. On the other hand, overexpressing c-Src (as is the case in SW480 Src531), increased tyrosine phosphorylation of Par-4. This indicates that Src is not the tyrosine kinase that is involved in phosphorylating the tyrosine site of Par-4. Nevertheless, c-Src may promote the tyrosine phosphorylation of Par-4 by another tyrosine kinase.

In this chapter, it has been shown that **the inhibition of c-Src in colon cancer in combination with 5-FU can activate endogenous Par-4 and cause cell death.** Par-4 was activated in colon cancer cells that were treated with a Src inhibitor and 5-FU. This was activation was seen in three ways: first, the interaction between Par-4 and Akt was reduced; second, the interaction between Par-4 and 14-3-3, specifically the ζ isoform, was decreased; and third, the localization of Par-4 to the nucleus was increased. These results demonstrate that activation of endogenous Par-4 may be involved in the cell death that has been observed with Src inhibition and 5-FU treatment.
Chapter 4

Summary and Future Directions

Par-4 has been shown to have potential as a pro-apoptotic molecule in a number of cancers. At the start of this dissertation work, however, there was only one publication reporting the involvement of Par-4 in colon carcinoma. HCA-7 colon carcinoma cells were treated with cyclooxygenase inhibitors, resulting in upregulation of Par-4 expression (337). This upregulation occurred under conditions that also caused apoptosis. From the research reported in this dissertation, it can be concluded that **increasing the pro-apoptotic activity of Par-4 in colon cancer results in cell death.**

Two specific aims were pursued. The first aim was to determine the impact of overexpressing Par-4 on cell death in colon cancer. Through the work reported here, it has been shown that Par-4 overexpression increases apoptosis in response to the chemotherapeutic agent 5-FU *in vitro*. In addition, Par-4 overexpression reduces tumor growth *in vivo*. The second aim of this dissertation research was to elucidate how endogenous Par-4 can be activated in colon cancer, resulting in cell death. In the work described here, it has been shown that Src inhibition and 5-FU treatment activate Par-4. The inhibitory interactions of Par-4 with Akt and 14-3-3σ were reduced. In addition, increased mobilization to the nucleus of Par-4 was observed with Src inhibition and 5-FU treatment. These results demonstrate that Par-4 overexpression is a promising strategy for colon cancer therapy. Additionally, Par-4 activation may play a role in the efficacy of chemotherapeutic regimen for colon cancer.
There remains a need to develop more effective therapies for colon cancer. Par-4 has been shown to induce apoptosis in different cancers. Given these facts, it has been the long-term goal of our research group to develop treatment regimens for colon cancer that exploit the pro-apoptotic activity of Par-4. With this dissertation work, the first steps toward the attainment of this long-term goal have been taken. The key issue that needed to be established was whether Par-4 overexpression induces apoptosis in colon cancer. Through the results reported here, it has been shown that Par-4 overexpression in combination with the apoptotic and chemotherapeutic agent 5-FU can cause cell death in colon cancer. In addition, it has been observed that tumors formed by Par-4 overexpressing colon cancer cells grew more slowly than those formed by wild type cells. As an extension of the experiments reported here (442), Par-4 has been overexpressed in vivo by injecting intravenously nanoliposome particles containing the pCB6+-par-4 plasmid (hereafter referred to as par-4 nanoliposome). Mice that received the par-4 nanoliposome and 5-FU treatment had tumors that grew more slowly than tumors from mice that receive ghost nanoliposome (i.e. contain no plasmid). Par-4 overexpression can be achieved in vivo not only by intravenous but also by intratumoral injection of the par-4 nanoliposome (442). The results from this related study and this dissertation work demonstrate that increasing Par-4 in colon cancers in vivo can inhibit the growth of tumors formed by colon cancer cells.

The research reported here has brought about new questions that need to be addressed in the pursuit of our long-term goal of using Par-4 as part of colon cancer therapy. In this body of work, the impact of Par-4 on proliferation of colon cancer cells
has been shown. Par-4 inhibited cell proliferation *in vivo*. Nevertheless, the effect of Par-4 on cell proliferation needs to be elucidated further. Although it has been proposed that Par-4 reduces proliferation primarily through the inhibitory activity of Par-4 on PKCζ (311), this may not be the primary mechanism in colon cancer cells. PKCζ expression is low in colon carcinoma cells (456-458). One mechanism by which cell proliferation can be decreased is by inhibition of cell cycle progression. Par-4 overexpression has been shown to inhibit cell cycle progression by inducing S-phase arrest in an immortalized epithelial cell line. The interaction of Par-4 with DNA topoisomerase 1 has been implicated in the S-phase arrest seen with Par-4 overexpression (241). The impact of Par-4 on the cell cycle in cancer cells, especially in colon cancer, has not been studied as extensively as the effect of Par-4 on cell death. If Par-4 also inhibits Top1 in colon cancer cells, then the potential of Par-4 in colon cancer therapy becomes more evident. One of the chemotherapeutics used in the clinic for colon cancer, irinotecan, is a DNA topoisomerase inhibitor (90, 459). However, because irinotecan primarily acts on DNA replication (91), it also affects actively dividing cells. Thus, side effects occur (460, 461). If Par-4 can be activated in colon cancer cells, and if Par-4 can inhibit DNA topoisomerase I (Top1) only in colon cancer cells, then side effects can be avoided. Thus, the effect of Par-4 on Top1 in the context of colon cancer is worth investigating.

Par-4 associates with different proteins, including Akt1, 14-3-3, Top1 and K-Ras effector RASSF2, in cancers (236, 241, 272, 331). Par-4 interacts with Akt1, 14-3-3σ, and NFκB in HT29 colon cancer cells (315). In this dissertation work, it has been shown that Par-4
also associates with c-Src. This interaction correlates with Src kinase activity and Akt1 phosphorylation at the S473 site. Inhibiting the association of Par-4 with c-Src, Akt and 14-3-3σ, however, was not enough to cause mobilization of Par-4 to the nucleus. This indicates that there are other factors aside from the proposed cytoplasmic sequestration of Par-4 by 14-3-3σ that keep Par-4 in the cytoplasm. The regulation of the movement of Par-4 to the nucleus needs further investigation. Par-4 has been shown to interact with the K-Ras effector RASSF2. One mechanism by which Par-4 moves to the nucleus is its association with RASSF2. RASSF2 interacts with the nuclear transport protein importin and enables the mobilization of RASSF2 partner proteins to the nucleus (333). However, this may not be a dominant mechanism in colon cancer because RASSF2 has been found to be downregulated in colon cancer. The RASSF2 promoter is hypermethylated in colon cancer. Thus, RASSF2 mRNA expression is decreased or even absent in colon cancer (462). This suggests that Par-4 is not mobilized to the nucleus by RASSF2.

Par-4 is phosphorylated on tyrosine residues. However, inhibiting Src kinase activity did not result in a decrease in tyrosine phosphorylation of Par-4. This suggests that Par-4 is not a direct kinase substrate of c-Src. Overexpressing c-Src, however, promoted the tyrosine phosphorylation of Par-4. The impact of the tyrosine phosphorylation on Par-4 activity needs to be investigated. This is particularly important in colon cancer, where increasing c-Src levels are typical in the more advanced stages of disease, including in metastases (340).
The survival rates of patients with colon cancer decrease with disease stage. As colon cancer progresses, gene and protein expression changes have been observed (463, 464). Resistance to apoptosis may increase as colon cancer progresses. The cell line used in most of the studies in this dissertation work was HT29. The HT29 cell line is derived from a human colon adenocarcinoma (465). However, given the differences in primary and metastatic colon cancer cells, it is important to investigate whether Par-4 overexpression can promote apoptosis in metastatic cells, as well. Future experiments can be performed with SW480 and SW620 cell lines. SW480 and SW620 cell lines have been derived from the primary tumor and lymph node metastases, respectively, of a patient (466). Thus, these two cell lines are genetically similar. SW620 cells are more resistant to extrinsic and intrinsic apoptotic mechanisms. This may be in part due to altered expression of proteins involved in the apoptotic pathway, namely, Fas, Apaf-1, XIAP, survivin, and AIF (208).

In this dissertation work and in recently published in vivo studies of Par-4 in colon cancer (442, 467), subcutaneous xenografts of HT29 colon cancer cells were tested. Although these studies are helpful in elucidating the potential of Par-4 in colon cancer in vivo, they have limitations. Colon cancer tumorigenesis, up to metastases, result not only from factors inside colon cancer cells but also from surrounding stroma and immune cells. These interactions are not modeled well in a subcutaneous xenograft. Therefore, the impact of Par-4 overexpression in other in vivo models of colon cancer needs to be assessed. The mortalities associated with colon cancer are predominantly from occurrence of metastases. Thus, the effects of Par-4 overexpression in an animal
model where primary colon cancer cells can metastasize need to be studied. An orthotopic transplantation model has been used to study primary tumor and metastatic growth of colon cancer cells (468, 469), and can be considered for future in vivo Par-4 studies.

The potential of Par-4 in colon cancer therapy has been demonstrated in this dissertation. Work remains to be done on the role that tyrosine phosphorylation of Par-4 plays in regulation of Par-4 activity. It has been reported here that Src inhibition and 5-FU treatment result in mobilization of Par-4 to the nucleus. The steps that occur after release of Par-4 from c-Src, Akt1, and 14-3-3 sigma, leading to the localization of Par-4 to the nucleus, have yet to be identified. This dissertation, however, does not only open up more basic questions but it also brings up questions of more immediate clinical relevance. Given that current chemotherapy regimen combine 5-FU with leucovorin/folinic acid and oxaliplatin (FOLFOX regimen), the question is whether Par-4 increases response to FOLFOX. The combination of the Src inhibitor PP2 with 5-FU has been shown here to activate Par-4 in vitro. Tests on whether the Src inhibitor in clinical trials (saracatinib (470)), in combination with 5-FU, can activate Par-4 in vivo are warranted. Although the groundwork has been laid and the potential of Par-4 in colon cancer therapy has been demonstrated, much remains to be done before Par-4 can be used in the arsenal against colon cancer. Nevertheless, the first steps have been taken. The hope is that one day colon cancer patients would be cured without having to endure side effects.
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Appendix A. Identification of Outlier Values Based on Median Absolute Deviation
(http://rfd.uoregon.edu/files/rfd/StatisticalResources/outl.txt)

A non-parametric or distribution-free approach to detect outliers is based on computing medians.

Steps:

1. The median of the original data is calculated.
2. The absolute value of deviations of original data from the median is computed.
3. The median of these absolute deviations is calculated.
4. The ratio of absolute deviation from step 2 and median from step 3 is computed.
   If this ratio is greater than 2.5, the data is considered an outlier.

The method is illustrated as follows:

Data (x) below is sorted from smallest to largest:

x  8 25 35 41 50 75 75 79 92 129

The median of these 10 values of x is 62.5.

The absolute values of the deviation (abs_dev) of original data from the median (med) are as follows:

<table>
<thead>
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<th>x</th>
<th>med</th>
<th>abs_dev</th>
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<tbody>
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</tr>
<tr>
<td>129</td>
<td>62.5</td>
<td>66.5</td>
</tr>
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</table>
The absolute values of the deviation are divided by the median of the absolute values:

<table>
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<th>x</th>
<th>Median</th>
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<th>Med of abs dev</th>
<th>abs_dev/Med</th>
<th>Outlier?</th>
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</tr>
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</table>

If the abs_dev/Med > 2.5, then the observed value is considered an outlier. According to this cutoff value, the data above includes one outlier (x=129).
Appendix B. MTT Viability Assay Results used to determine the appropriate 5-FU concentration to be used for assessing apoptosis in SW480 mock and SW480 Src531 clones. The horizontal line in the middle of the graph denotes fold change in viability (versus SW480 mock) equal to 0.5.
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