

**MECHANISMS OF THERAPY-RESISTANCE MODULATED BY GRP78, SPARC AND
HSP47 IN COLORECTAL CANCER**

by

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Abstract

Colorectal cancer (CRC) is the third leading cause of cancer mortality worldwide. The primary cause of treatment failure is the emergence of drug resistance in CRC following chemotherapy. Therefore, understanding the molecular mechanisms underlying chemoresistance in CRC is necessary for the development of effective anti-cancer therapy. We previously identified matricellular protein SPARC as a chemosensitizing agent that promotes apoptosis in CRC chemotherapy. To further understand the mechanisms by which SPARC exerts its chemosensitizing functions in CRC cells, we conducted immunoprecipitation followed by tandem mass spectrometry (MS/MS) analysis and identified GRP78- an endoplasmic reticulum (ER)- resident stress modulator- as a putative binding protein of SPARC. We found that SPARC is able to sensitize CRC cells under chemotherapy to ER stress-associated death by lowering the activation threshold of ER stress signaling via its interaction with GRP78. Notably, it was observed that a relatively higher ratio of GRP78 to SPARC expression correlated to shorter overall survival in patients with CRC, suggesting this finding is relevant clinically. Another SPARC-binding protein identified in the MS/MS analysis was ER-resident protein HSP47. As HSP47 is mostly unexplored in cancer, we focused on understanding the biology behind the role of HSP47 in CRC, especially its effects in relation to the chemotherapeutic response. We found that HSP47 is upregulated in CRC human tissues, and the expression of HSP47 promotes the resistance in CRC cells exposed to 5-fluorouracil (5-FU) by inhibiting apoptosis. In addition, overexpression of HSP47 also enhances Akt signaling in CRC cells exposed to chemotherapy by decreasing the protein stability of the Akt-specific phosphatase PHLPP1. Importantly, this finding was also confirmed *in vivo* as tumors with HSP47 overexpression were shown to be more resistant to 5-FU exposure and have elevated level of Akt activation. Taken together, our studies

suggest a complex protein network surrounding SPARC that modulates the treatment response of CRC following chemotherapy. Therapeutic approaches against HSP47 or targeting GRP78 via SPARC mimetics may improve chemotherapeutic efficacy in CRC.

Lay Summary

The primary cause of treatment failure in cancer therapy is drug resistance, which renders cancer cells non-responsive to chemotherapeutic agents. It is important to understand the underlying mechanisms in order to develop more effective treatment strategies. Colon and rectum cancer (CRC) is the third most commonly diagnosed cancer worldwide. This thesis focuses on how three proteins, SPARC, GRP78 and HSP47, affect the response of CRC cells to chemotherapy and the underlying mechanisms. I found that SPARC makes CRC cells more sensitive to the stress generated inside cells exposed to chemotherapeutic drugs by interacting with GRP78. This effect will eventually lead to cancer cells death. On the other hand, HSP47 expression increases in CRC and it promotes drug resistance by enhancing survival signaling. Overall, this study shows that SPARC, GRP78 and HSP47 are involved in chemoresistance and drugs modulating them might increase the chemotherapeutic efficacy in CRC.

Preface

Tumor tissues were obtained from patients through a protocol approved by the Clinical Research Ethics Board of the University of British Columbia (UBC) and the BC Cancer Agency (BCCA). All patients signed a consent form approved by the Ethics Board (UBC Ethics Board #: H14-02808). Animal care and experimental procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care (CCAC) under the approval of the Animal Care Committee of the University of British Columbia (protocol #: A16-0092).

A version of Chapter 3 has been accepted by *Cell Death & Disease*. [Yi-Jye Chern], John C.T. Wong, Grace S.W. Cheng, Angel Yu¹, Yaling Yin, David F. Schaeffer, Hagen F. Kennecke, Gregg Morin, Isabella T. Tai. *The interaction between SPARC and GRP78 interferes with ER stress signaling and potentiates apoptosis via PERK/eIF2 α and IRE1 α /XBP-1 in colorectal cancer*. I am the lead investigator of this study. I wrote the manuscript, conducted experiments, data analysis and interpreted the results. Grace S.W. Cheng conducted the mass spectrometry analysis in this thesis in Table 3.1; Angel Yu performed the immunoprecipitation assay in Fig. 3.1.B; John C.T. Wong and Yaling Yin conducted the IHC assay and statistical analysis in Fig. 3.7. Dr. Hla-Win Piazza assisted with the MTS and TUNEL assay in Fig. 3.5. Isabella T. Tai supervised the study.

A version of Chapter 4 is under preparation for manuscript submission. [Yi-Jye Chern], Peter Zhang, Hye-Lim Ju, Isabella T. Tai. *Heat shock protein 47 promotes tumor survival and therapy resistance by modulating AKT signaling via PHLPP1 in colorectal cancer*. I am the lead investigator of the study. I wrote the manuscript, conducted experiments, data analysis and interpreted the results. Peter Zhang performed the bioinformatic analysis in Fig. 4.1.B, C and 4.2, and Hye-Lim Ju assisted with the animal work in Fig. 4.16. Isabella T. Tai supervised the study.

Table of Contents

Abstract.....	iii
Lay Summary	v
Preface.....	vi
Table of Contents	vii
List of Tables	xi
List of Figures.....	xii
List of Abbreviations	xv
Acknowledgements	xvii
Dedication	xix
Chapter 1: Introduction	1
1.1 Colorectal Cancer (CRC).....	1
1.1.1 Overview/ Etiology/ Epidemiology	1
1.1.2 Colon and Rectum.....	2
1.1.3 Molecular Aberration in CRC.....	4
1.1.3.1 PI3K/Akt signaling	6
1.1.3.1.1 PHLPP.....	9
1.1.4 Clinical Management	10
1.1.4.1 Screening, Diagnosis and Staging.....	10
1.1.4.2 CRC treatment	11
1.2 Chemoresistance	12
1.2.1 Overview.....	12
1.2.2 Chemotherapeutic Drug- Specific Chemoresistance	13

1.2.3	Targeted Therapy Drug Resistance.....	14
1.2.4	Stress Response in the Chemoresistance	15
1.2.4.1	Overview.....	15
1.2.4.2	ER Stress signaling.....	17
1.3	SPARC.....	20
1.4	GRP78.....	21
1.5	HSP47	22
1.6	Thesis Theme and Rationale.....	23
1.7	Hypotheses and Specific Aims	24
Chapter 2: Materials and Methods		26
2.1	Parental and Resistant Cell Lines	26
2.2	Immunoblotting.....	26
2.3	Immunofluorescence Analysis.....	27
2.4	Q-RT-PCR	27
2.5	MTS Cell Viability Assay.....	28
2.6	TUNEL Assay.....	28
Chapter 3: The Interaction between SPARC and GRP78 Interferes with ER Stress		
Signaling and Potentiates Apoptosis via PERK/eIF2α and IRE1α/XBP-1 in Colorectal		
Cancer		29
3.1	Introduction.....	29
3.2	Materials and Methods.....	30
3.2.1	Mass Spectrometry Analysis.....	30
3.2.2	RT-PCR Assay for XBP-1 Splicing Detection	31

3.2.3	Cell Fractionation and Immunoprecipitation	31
3.2.4	RNA Interference.....	32
3.2.5	Colocalization Analysis	33
3.2.6	Tissue Microarray (TMA) construction and Immunohistochemistry (IHC)	33
3.2.7	Statistics	34
3.3	Results.....	35
3.3.1	A Novel Interaction between SPARC and GRP78 Occurs in CRC.....	35
3.3.2	A Dynamic Interaction between SPARC and GRP78 in the ER Occurs in the Presence of ER Stress	39
3.3.3	GRP78 Reduces SPARC's Pro-apoptotic Effects During ER Stress.....	43
3.3.4	Low Expression of GPR78 to SPARC ratio Correlates with Improved Survival in Individual with CRC	47
3.3.5	SPARC Sensitizes CRC to PERK/eIF2 α and IRE1 α / XBP-1 ER Stress Signaling .	49
3.3.6	SPARC Modulates ER Stress Signaling through Its Interference in the Binding between ER Stress Sensor and GRP78	55
3.4	Discussion	58
 Chapter 4: Heat Shock Protein 47 Promotes Chemoresistance by Modulating AKT Signaling via PHLPP1 in Colorectal Cancer.....62		
4.1	Introduction.....	62
4.2	Materials and Methods.....	63
4.2.1	Patients Cohort and Data Analysis	63
4.2.2	Plasmids Construction and Stable Cell Line Establishment	63
4.2.3	Lentiviral Infection	64

4.2.4	Immunoprecipitation.....	65
4.2.5	Xenograft Study	65
4.3	Results.....	66
4.3.1	Increased HSP47 Expression in the Tumors of CRC Patients.....	66
4.3.2	HSP47 Expression Promotes the Resistance of CRC Cells to Chemotherapy	72
4.3.3	HSP47 Inhibits the Apoptosis Induction of CRC Cells under Chemotherapy	76
4.3.4	HSP47 Promotes the Phosphorylation of AKT at Ser 473 in CRC Cells under Chemotherapy	78
4.3.5	HSP47 Interacts with PHLPP1 and Decreases Its Protein Stability	81
4.3.6	HSP47 Promotes Tumor Survival under Chemotherapy via AKT Activation in CRC Xenograft Model.....	84
4.4	Discussion.....	87
Chapter 5: Conclusion.....		90
5.1	Summary of Study and Findings	90
5.1.1	Chapter 3.....	90
5.1.2	Chapter 4.....	90
5.2	Conclusions Regarding the Study Hypotheses	91
5.3	Strengths and Limitations	92
5.4	Overall Significance and Clinical Implications	94
5.5	Future Research Directions.....	94
References.....		97

List of Tables

Table 3.1 Immunoprecipitation- Mass Spectrometry Identification of SPARC Interacting Proteins	37
Table 4.1 Association between HSP47 expression and clinic pathological factors in the TCGA cohort	69
Table 4.2 Association between HSP47 expression and clinic pathological factors in the curatedCRCData cohort	70

List of Figures

Figure 1.1 The anatomy of colon and rectum	3
Figure 1.2 Model of Akt kinase activation	8
Figure 1.3 ER stress signaling activation.....	19
Figure 3.1 Interaction between GRP78 and SPARC in CRC	38
Figure 3.2 Increased interaction between GRP78 and SPARC in the membranous fractions of HCT116 cells under 5-FU and CPT-11-induced ER stress	40
Figure 3.3 Increased colocalization between GRP78 and SPARC in HCT116 cells under CPT-11 treatment	41
Figure 3.4 Colocalization between GRP78 and SPARC in the perinuclear region of SPARC-overexpressing MIP/SP exposed to ER stress-inducer tunicamycin	42
Figure 3.5 GRP78 overexpression increases the cell viability and suppresses apoptosis induction in SPARC-overexpressing MIP/SP under the treatment of 5-FU and ER stressor tunicamycin ..	45
Figure 3.6 CRC cell lines with high relative expression levels of GRP78 to SAPRC are more resistant to drugs	46
Figure 3.7 CRC patients with a low ratio of GRP78:SPARC protein expression have improved prognosis.....	48
Figure 3.8 SPARC promotes early activation of PERK-eIF2 α and IRE1 α -sXBP ER stress signaling in endogenous HCT116 and MIP/SP cells.....	50
Figure 3.9 Knockdown of SPARC in HCT116 cells suppresses the activation of ER stress signaling induced by CPT-11 treatment	51
Figure 3.10 SPARC overexpression in MIP101 cells promotes early activation of PERK/eIF2 α ER stress signaling.....	52

Figure 3.11 SPARC overexpression in MIP101 cells promotes early activation of IRE1 α /XBP-1 ER stress signaling.....	53
Figure 3.12 SPARC overexpression in MIP101 cells promotes apoptosis and autophagy activation.....	53
Figure 3.13 5-FU-induced ER stress and apoptosis in HCT116 and MIP/SP	54
Figure 3.14 SPARC interferes with the binding between GRP78 and PERK under ER stress	56
Figure 3.15 SPARC reduces the tolerance to ER stress in CRC cells	57
Figure 4.1 Increased expression of HSP47 in colorectal cancer.....	68
Figure 4.2 CRC patients with high HSP47 expression level have lower overall survival.....	71
Figure 4.3 HSP47 protein expression in CRC cell lines	73
Figure 4.4 Examination of HSP47 expression level in CRC cell lines with HSP47 transient overexpression or knockdown	73
Figure 4.5 Transient knockdown of HSP47 sensitized RKO and CCL228 CRC cell lines toward chemotherapy.....	74
Figure 4.6 Transient expression of HSP47 increases the resistance of RKO, CCL228 and HCT116 CRC cell lines under chemotherapy	74
Figure 4.7 Increased HSP47 mRNA and protein expression in CRC resistant cell lines	75
Figure 4.8 HSP47 expression suppresses apoptosis activation in HCT116 exposed to 5-FU in the TUNEL assay.....	77
Figure 4.9 HSP47 expression suppresses the cleavage of caspase-3 and -7 in HSP47-overexpressing and knockdown cell lines.....	77
Figure 4.10 HSP47 promotes Akt activation and reduces PHLPP1 expression in CRC cells exposed to 5-FU.....	79

Figure 4.11 Quantification of immunoblotting images of p-Akt (S473) in HCT116/HSP47 and RKO/si-HSP47 exposed to 5-FU	79
Figure 4.12 Quantification of immunoblotting images of PHLPP1 in HCT116/HSP47 and RKO/si-HSP47 exposed to 5-FU	80
Figure 4.13 HSP47 mRNA expression in HCT116/HSP47 and RKO/si-HSP47 exposed to 5-FU	82
Figure 4.14 HSP47 expression reduces PHLPP1 protein stability in cycloheximide chase assay	82
Figure 4.15 HSP47 colocalizes and interacts with PHLPP1 in RKO CRC cell line	83
Figure 4.16 HSP47-overexpressing CRC tumors have larger tumors despite treatment with chemotherapy	85
Figure 4.17 HSP47 promotes chemoresistance in CRC through enhancing Akt activation.....	86

List of Abbreviations

5-FU	5-fluorouracil
AJCC	American Joint Committee on Cancer
Akt	v-Akt Murine Thymoma Viral Oncogene
ATCC	American Type Culture Collection
ATF6	Activating transcription factor 6
CHOP	CCAAT/-enhancer-binding protein homologous protein
CIN	Chromosomal instability
Co-IP	Co-immunoprecipitation
CPT-11	Camptosar (i.e. Irinotecan)
CRC	Colorectal cancer
DDR	DNA damage response
DPD	Hydropyrimidine dehydrogenase
ECL	Enhanced chemiluminescence
eIF2a	Eukaryotic translation initiator factor-2 a
ER	Endoplasmic reticulum
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
FFPE	Formaldehyde and paraffin embedded
GFP	Green fluorescent protein
GPCR	G-Protein-Coupled Receptor
GRP78	Glucose-regulated protein, 78kDa
HNPCC	Hereditary nonpolyposis colorectal cancer
HSP47	Heat shock protein 47
IHC	Immunohistochemistry
IP	Immunoprecipitation
IRE1a	Inositol-requiring enzyme 1 a
LOH	Loss of heterozygosity
MAP	MYH-associated polyposis
MAPK	Mitogen-activated protein kinase
mCRC	Metastatic colorectal cancer
MRP	Multidrug resistance protein
MSS	Microsatellite stable
MSTF	Multi-Society Task Force
mTOR	Mammalian target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NCS	Newborn calf serum
PAM	PI3K/Akt/mTOR

PERK	Protein kinase RNA-like ER kinase
PHLPP	PH-domain leucine-rich-repeat-containing protein phosphatase
PI3K	Phosphoinositol 3-kinase
PIP2	Phosphatidylinositol-3,4-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-Triphosphate
PKB	Protein Kinase-B
PTEN	PI3K inhibitor phosphatase and tensin homolog
Q-RT-PCR	Quantitative reverse transcription polymerase chain reaction
ROC	Receiver operating characteristic
RT	Room temperature
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
SPARC	Secreted protein acidic and rich in cysteine
TCGA	The Cancer Genome Atlas
TMA	Tissue microarray
TMN	Tumor depth, nodal metastasis, and distant metastasis
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick ending labeling
XBP-1	X-box binding protein 1

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Dedication

In Remembrance Of

Dr. KONAN PECK

Died 22nd Dec 2011

I still and will always remember you, my beloved research mentor. Thank you for inspiring me to pursue sciences and even to find the meaning of human life with your own life.

Chapter 1: Introduction

1.1 Colorectal Cancer (CRC)

1.1.1 Overview/ Etiology/ Epidemiology

Colorectal cancer (CRC) is the third most commonly diagnosed cancer among males and second among females in the world (1). For an average-risk person, defined as an individual without a personal or family history of CRC and above the age of 50, the lifetime risk of CRC is 5%- 6% (2). Most cases of CRC are sporadic. Risk factors include increasing age, male sex, colonic polyps history, and environmental factors, such as red meat, high-fat diet, inadequate intake of fiber, overweight, sedentary lifestyle, tobacco use, alcohol consumption and diabetes mellitus (3). Patients with inflammatory bowel diseases (ulcerative colitis and Crohn's disease) also have increased risk of CRC (4,5).

It is estimated that approximately 20%-30% of all CRC have familial basis (defined by two or more first-degree relatives with this cancer) (2). Familial adenomatous polyposis (FAP), hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome and *MYH*-associated polyposis (MAP) are hereditary colon cancer with defined genetic defects. These colon cancers with hereditary syndromes account for nearly 6% of CRC cases- namely, 3%-4% of those with HNPCC, nearly 1% with FAP and less than 1% of cases are due to MAP (2). It has been identified that the primary genetic defects for these inherited CRC are the germline mutations in DNA mismatch repair (MMR) genes (such as *MLH1*, *MSH2*, *MSH6* and *PMS2*) for HNPCC, *APC* tumor suppressor gene or Wnt signaling pathway for FAP, and *MYH* base excision repair gene in MAP (2,6).

The mortality rate of CRC has been decreasing in most countries in recent years because of the dissemination of screening test, reduced prevalence of risk factors, and/ or improved

treatment. However, the survival rate of CRC patients diagnosed with distant metastasis remained only 14% (7). Among the CRC patients, approximately 25% present with distant tumors at initial diagnosis and almost 50% of patients will develop metastases, indicating an urgent need for the development of effective chemotherapy for metastatic CRC (mCRC).

1.1.2 Colon and Rectum

The intestine, which is made up of the small and large intestine, is the last part of the digestive system in the human body. The large intestine consists of four parts: cecum, colon, rectum and anal canal. The function of the colon is to absorb the water, electrolytes and key nutrients from the partly digested foods passed from the cecum, which is connected to the small intestine. The remaining materials, solid waste called stool, move through the colon and is temporarily stored in the rectum until it passes out of the body through anal canal and anus. The colon is a U-shaped tube, which can be divided into four parts: ascending colon, transverse colon, descending colon, and sigmoid colon. Primary tumors derived from different parts of the colon are molecularly and clinically distinct (8,9). Left-sided tumors, including those developing in the splenic flexure (the sharp bend between the transverse colon and descending colon), descending colon, sigmoid colon, rectum or distal third of the transverse colon, originate from the embryonic hindgut, while right-sided tumors, including cancer of the cecum, the ascending colon up to the hepatic flexure (the sharp bend between ascending colon and transverse colon) or the proximal two-thirds of the transverse colon, derive from the embryonic midgut. Left-sided tumors are more often characterized by EGFR inhibitor-sensitive phenotypes (ie, *EGFR* amplification, epiregulin high, and chromosomal instability). In contrast, right-sided tumors more frequently possess a host of adverse prognostic factors, including *BRAF* mutation,

microsatellite instability (prognostic in stage IV disease), hypermutation, and mucinous histology (10). These differences are also translated into differential clinical outcomes, with right-sided tumors commonly associated with poor prognosis (11-13).

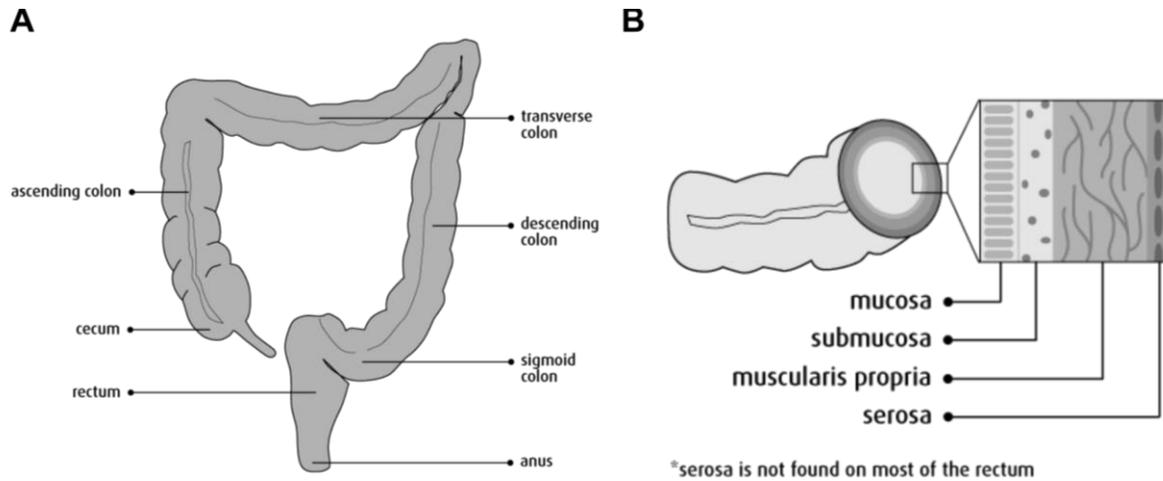


Figure 1.1. The anatomy of colon and rectum. (A) The anatomy of colon and rectum. (B) The layers of the colon and rectum. Permission to use the images has been obtained from Canadian Cancer Society. (<http://www.cancer.ca/en/cancer-information/cancer-type/colorectal/colorectal-cancer/the-colon-and-rectum/?region=on>)

1.1.3 Molecular Aberration in CRC

CRC develops via an adenoma-carcinoma sequence, which describes the stepwise progression from normal to dysplastic glandular epithelium to carcinoma associated with the accumulation of multiple clonally selected genetic alterations (14). Molecular analysis of primary tumors can classify CRC by the types of mutations or epigenetic changes which accumulate genome-wide during carcinogenesis (15). It has been shown that most of the CRC are aneuploid, demonstrating frequent deletions of chromosome 5q, chromosome 17 and 18 as well as gains of chromosome 1q, 7p and q, 8p and q, 13q, and 20p and q (16). These tumors display high chromosomal instability (CIN) but microsatellite stable (MSS) phenotype, and account for approximately 85% of sporadic CRC (15). By contrast, the remaining cases (about 15%) have no apparent allelic loss of heterozygosity (LOH) but shows a high frequency of microsatellite instability (MSI-H) owing either to epigenetic silencing of *MLH1* or mutations in one of the mismatch repair genes *MLH1*, *MSH2*, *MSH6* or *PMS2* (17). Accumulating evidence indicates that CRC with CIN or MSI phenotypes may demonstrate different pathology features, drug response and prognosis following chemotherapy (18-24).

CRC acquires numerous genetic changes, but certain signaling pathways are particularly essential in tumor progression. For example, the activation of the Wnt signaling pathway is considered as the initiating event in the adenoma-carcinoma sequence. Approximately 80% of sporadic colorectal adenomas and carcinomas have somatic mutations that inactivate tumor suppressor gene *adenomatous polyposis coli* (*APC*) (25). Mutations in *APC* are one of the earliest mutations in colon cancer progression. The dysfunctional *APC* fails to regulate cytosolic levels of the signaling molecule β -catenin, resulting in constitutively activated WNT signaling (25). Oncogenic mutations of the Ras family of small-G proteins are also common targets for

somatic mutations in CRC. Approximately 40% of patients have *KRAS* somatic mutations, most of which affect codons 12, 13 and 61 (26,27). Mutant *KRAS* alleles are not required for adenoma initiation; however, when present, they appear to drive the behavior of advanced CRC cells (26). The RAS proteins exert their effects mainly by activating downstream signaling such as mitogen-activated protein kinase (MAPK) signaling, via RAF, and phosphoinositol 3-kinase (PI3K) /Akt signaling, via PI3K. The activated RAF protein signals through its serine-threonine kinase activity, which further activate MAPK effectors MEK1 and MEK2. The gene encoding BRAF is mutated in approximately 5-10% of CRCs, and is found to be mutually exclusive to *KRAS* mutation (28). On the other hand, *PIK3CA*, the catalytic p110 α subunit of lipid kinase PI3K, and PI3K inhibitor phosphatase and tensin homolog (*PTEN*), are found mutant in approximately 10~15% (27,29) and ~20% of CRCs with either wild-type or mutant *KRAS* (30,31), respectively. This suggests there may be a synergistic effect in promoting tumor progression in cells with *KRAS* mutation and activated PI3K/Akt signaling.

In addition to *APC*, tumor suppressor gene *TP53* is also frequently inactivated in CRC. *TP53* is usually inactivated by a combination of 17p chromosomal deletion (~75% of CRCs) and a missense mutation in the remaining allele (~85% of CRCs) (32-34). Wild-type p53 mediates cell-cycle arrest and apoptosis in cells under stress. In CRC, the inactivation of p53 often coincides with the transition of large adenomas into invasive carcinoma, indicating that p53 mutations may be a rate-limiting step for the continued growth and acquisition of invasive properties of tumors (34). The mutational inactivation of the transforming growth factor- beta (TGF- β) tumor-suppressor pathway is also frequently seen during the transition from human colon adenoma to malignant carcinoma (35). Inactivating mutations in the TGF- β type II receptor (*TGF β IIR*) gene are found in approximately one-third of CRCs (36,37), while the two

downstream proteins SMAD4 and SMAD2 are inactivated in ~10-15% and ~5% of CRCs, respectively (27,38).

1.1.3.1 PI3K/Akt signaling

Akt (v-Akt Murine Thymoma Viral Oncogene)/ PKB (Protein Kinase-B) is a serine/threonine kinase that is involved in modulating numerous biological activities, such as cell proliferation, cell metabolism and the inhibition of apoptosis. The Akt kinase family is composed of three highly homologous isoforms: Akt1/PKB-alpha, Akt2/PKB-beta and Akt3/PKB-gamma. All three isoforms of Akt contain a common structure of three domains: PH (Pleckstrin Homology) domain at N-terminus, kinase domain in the central region, and regulatory domain at C-terminus (39). Various growth factors, cytokines and hormones activate Akt by binding their cognate receptor tyrosine kinase (RTK), cytokine receptor, or G-Protein-Coupled Receptor (GPCR) and induce autophosphorylation of tyrosine residues on the intracellular domain of the receptor. PI3K is then recruited to the phosphotyrosine residues on the receptor or the scaffold proteins and activated via conformational changes. Activated PI3K generates phosphatidylinositol-3,4,5-Triphosphate (PIP3) from phosphatidylinositol-3,4-bisphosphate (PIP2) at plasma membrane. Akt binds PIP3 through its PH domain and translocates to the membrane for activation. Akt is activated through a dual phosphorylation mechanism. 3-phosphoinositide-dependent protein kinase 1 (PDK1), which is also brought to the membrane through its PH domain, phosphorylates Akt at Thr308 in the “activation loop”, leading to partial Akt/PKB activation (40). Phosphorylation of Akt at Ser473 in the carboxy-terminal hydrophobic motif by mammalian target of rapamycin complex 2 (mTORC2) (41) induces full Akt activity. Akt can be negatively regulated by the dephosphorylation of Thr308 by protein phosphatase 2

(PP2A) (42), and Ser473 by the PH-domain leucine-rich-repeat-containing protein phosphatase (PHLPP1/2) (43,44), and the conversion of PIP3 to PIP2 by PTEN (45) (Fig. 1.1).

Akt promotes cell growth in part by phosphorylation of tuberous sclerosis complex 2 (TSC2), which, upon phosphorylation by Akt, relieves its suppression of Ras homolog enriched in brain (Rheb), which in turn activates the rapamycin-sensitive mTORC1 complex (46). mTORC1 phosphorylates proteins including S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), resulting in protein translation and lipid biogenesis (47). Cell proliferation is promoted by Akt's phosphorylation on cell cycle regulators p27 (48), while anti-apoptosis responses are regulated by its phosphorylation of members of the Forkhead box O (FOXO) family of transcription factor (49), and pro-apoptotic members of the Bcl-2 family of proteins such as Bad (50).

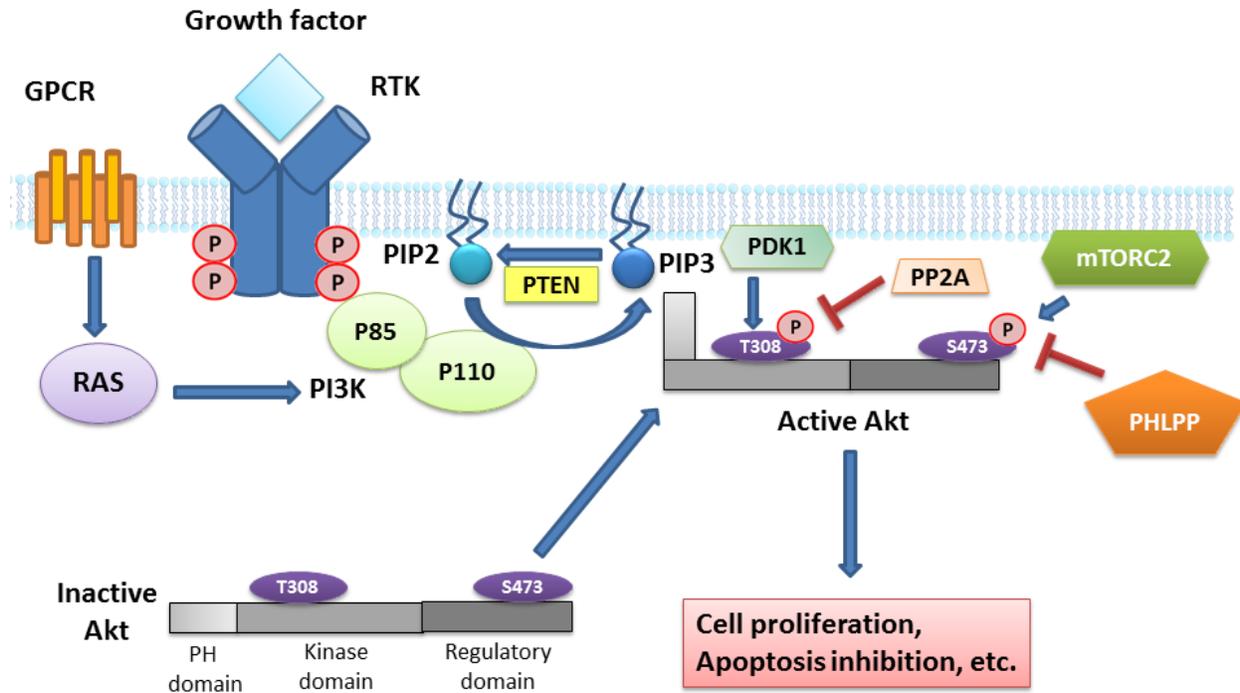


Figure 1.2. Model of Akt kinase activation. Ligand-mediated activation of receptor tyrosine kinase (RTK) or G-Protein-Coupled-Receptor (GPCR) promotes recruitment of phosphoinositide 3-kinase (PI3K) via its regulatory domain (p85) to the plasma membrane. This triggers the activation of PI3K and conversion by its catalytic domain (p110) of phosphatidylinositol-3,4-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-tris-phosphate (PIP3). Akt binds to PIP3 at plasma membrane via its PH domain, allowing 3-phosphoinositide-dependent protein kinase 1 (PDK1) to access and phosphorylate T308 in the “activation loop,” leading to partial Akt activation (40). Phosphorylation of Akt at S473 in the regulatory domain by mTORC2 stimulates full Akt activity (41), including inhibitory phosphorylation of the pro-apoptotic FOXO proteins (51). Dephosphorylation of T308 by protein phosphatase 2 (PP2A) (42), and S473 by PH-domain leucine-rich-repeat-containing protein phosphatase (PHLPP1/2) (44), and the conversion of PIP3 to PIP2 by phosphatase and tensin homolog (PTEN) (45) antagonize Akt signaling. Activated Ras proteins can also activate PI3K and the downstream Akt signaling.

1.1.3.1.1 PHLPP

The PH-domain leucine-rich-repeat-containing protein phosphatases (PHLPP), including PHLPP1 and PHLPP2, are a part of the protein phosphatase 2C (PP2C) family of Ser/Thr phosphatases (52). PHLPP1 has two predominant splice variants: PHLPP1 α and PHLPP1 β (43,44). PHLPP is evolutionary conserved. Both PHLPP1 and PHLPP2 share similar domain structures, including a predicted N-terminal Ras-association (RA) domain, a PH domain, a hydrophobic leucine-rich repeat (LRR) region, a catalytic Mn²⁺-dependent PP2C phosphatase domain, and a PDZ (postsynaptic density protein PSD95, *Drosophila* disc large tumor suppressor DLG1, and zonula occludens-1 protein zo-1) ligand (43). PHLPP was initially identified to dephosphorylate the hydrophobic motif of Akt at Ser473. It was later found to attenuate Akt activity in an isoform-specific manner: PHLPP1 targets Akt2 and Akt3 whereas PHLPP2 targets Akt1 and Akt3 (44). Interestingly, dephosphorylation at only the hydrophobic motif impairs the intrinsic catalytic activity of AKT by 90% (53). Moreover, although a subset of Akt substrates, such as TSC2 and GSK3, can still be phosphorylated when Akt is only phosphorylated at Thr308, phosphorylation of S473 of Akt is required to target FOXO1/3a, suggesting that the function of PHLPP as Akt-Ser473-specific phosphatase is critical in the control of cell survival (51,54). In addition to Akt, PHLPP also selectively dephosphorylates the hydrophobic motif of S6K and protein kinase C (PKC) to inhibit the protein translation and destabilize PKC, respectively (55,56). Moreover, by removing the inhibitory phosphorylation on Thr387, PHLPP also activates pro-apoptotic kinase mammalian sterile 20-like kinase 1 (Mst1) to induce apoptosis (57).

Given the function of PHLPP as a suppressor of Akt signaling and protein synthesis, as well as its ability to induce apoptosis, PHLPP is considered to curb tumor progression and its

role as a tumor suppressor *in vivo* is supported by several studies. First, PHLPP overexpression decreases colony formation and growth *in vitro* and in xenograft models in glioblastoma, colon, breast and pancreatic cancer (43,57-59). Second, loss or decrease of PHLPP expression was found in a variety of cancers (60). PHLPP1 gene is located on chromosome 18q21.33, a region that undergoes loss of heterozygosity in a high percentage of colon cancer (58,61). Immunohistochemistry study showed that the expression of PHLPP1 was lost or decreased in 78% of colorectal tumor tissues (58). Likewise, loss of heterozygosity is also common at the PHLPP2 locus, on chromosome 16q22.3, in breast cancer and hepatocellular carcinoma (62,63). Importantly, PHLPP1 knockout mice form neoplasia and, on partial Pten-loss, carcinoma in the prostate of mice (64). It was found that, in samples of prostate cancer patients, the co-deletion of PTEN and PHLPP1 is restricted to those with metastatic disease, suggesting a crucial role of PHLPP1 in inhibiting both tumorigenesis and metastasis (64).

1.1.4 Clinical Management

1.1.4.1 Screening, Diagnosis and Staging

CRC screening is a process that detects precancerous lesions or early-stage colorectal cancer in asymptomatic people without a history of cancer or precancerous lesions. Cancer prevention and earlier diagnosis through screening are considered to contribute to the improvements in the incidence and mortality of CRC in recent years (7). According to the recommendations from the U.S. Multi-Society Task Force of Colorectal Cancer (MSTF), for people with no family history of CRC, screening should start at age of 50 years (65). For individuals with a family history fulfilling the Amsterdam criteria, colonoscopy screening is recommended every two years starting at the age of 30 years. For those individuals with a family

history not fulfilling the Amsterdam criteria, colonoscopy screening is suggested to begin at age 40 years or 10 years younger than the affected relative, whichever is earlier (66,67). Different screening options are available. Fecal immunochemical test, which is noninvasive and low-cost, is recommended annually. Tests with high sensitivity but with invasive nature such as colonoscopy are recommended at 10-year interval in average-risk people (65). For patients with symptoms or positive screening tests, diagnostic examinations with colonoscopy is performed followed by imaging and histological tests to determine cancer stage (68). Tumor depth (T), nodal metastasis (N), and distant metastasis (M) (TNM) have been shown to be prognostic factors in CRC. Colon cancer staging is determined by combining the T, N and M classification according to the American Joint Committee on Cancer (AJCC)/ TNM system.

- **Stage 0:** The cancer cells are only limited in lamina propria. This is also called carcinoma in situ.
- **Stage I:** Tumor invades to submucosa or muscularis propria of the colon wall.
- **Stage II:** Tumor invades through muscularis propria into the pericolonic tissue but has not spread to nearby lymph node.
- **Stage III:** Regional lymph node metastasis has been detected.
- **Stage IV:** Cancer has spread to distant organs.

1.1.4.2 CRC treatment

The treatment plan for an individual diagnosed with CRC is based on several factors including the location of the tumor, the stage of the cancer, the general health and the preference of the individual. Surgery is the primary treatment for most CRC patients. To remove polyps or early-stage tumors that are on the surface of the lining of the intestine, local excision (local

resection) is performed. For most CRC, bowel resection that removes part of the intestine and nearby lymph nodes is required. Chemotherapy is commonly used for patients with stage II or III CRC after surgery. For patients with stage IV or recurrent cancer that cannot be removed by surgery, chemotherapy is the main treatment option. Chemotherapy drugs commonly used for CRC patients include 5-fluorouracil (Adrucil, 5-FU), irinotecan (Camptosar, CPT-11) and oxaliplatin (Eloxatin). Targeted drug therapy such as bevacizumab (against vascular endothelial growth factor, VEGF), cetuximab (against epidermal growth factor receptor, EGFR) and panitumumab (against EGFR) are sometimes included in the regimen to increase treatment efficacy (69). The selection of therapy depends on consideration of the goals of therapy, the type and timing of prior therapy, the mutational profile of the tumor, and the various toxicity profiles of the constituent drugs (70).

1.2 Chemoresistance

1.2.1 Overview

One of the challenges to successful chemotherapy is the emergence of drug resistance in cancer cells. Chemoresistance can be broadly categorized into two types: (1) intrinsic resistance and (2) acquired resistance. Intrinsic resistance refers to pre-existing factors in tumors before receiving chemotherapy that prevents an effective therapeutic response. Acquired resistance, on the other hand, refers to the resistance that develops in response to therapy in tumors that were initially sensitive toward treatment. Acquired resistance can develop through the emergence of mutation, the altered expression level of targeted proteins, or through a therapy-induced selection of a minor subpopulation from the originally heterogeneous tumors (71,72).

These two types of resistance share many common mechanisms, including reduced drug transport, altered drug targets and metabolism, deregulated DNA damage pathways, defective apoptotic signaling, activation of pro-survival signaling, protumorigenic tumor microenvironments, and stress adaptive responses (71). The kinds of resistance mechanisms adopted by a tumor depend on the regimens of the therapy, the tumor microenvironment, the intrinsic properties of the tumor such as the source organ, genetic and epigenetic profiles. At the level of tumors, different resistance mechanisms can act simultaneously to promote tumor survival. Understanding the molecular basis of these resistance mechanisms will facilitate drug development, rational cancer regimen design and patient selection to increase therapeutic efficacy.

1.2.2 Chemotherapeutic Drug- Specific Chemoresistance

Taking advantage of the fast-growing characteristic of cancer cells, the majority of traditional chemotherapeutic drugs inhibit tumor growth by targeting the cell-cycle machinery and inducing DNA damage. 5-FU has remained the mainstay of chemotherapeutic regimens for CRC therapy. The active metabolites of 5-FU disturb RNA synthesis and inhibit thymidylate synthase (TS), a nucleotide synthetic enzyme, that can also be directly misincorporated into DNA, causing DNA damage and cell death (73). However, the response rate to 5-FU as monotherapy is less than 15% (74). Although combination with therapeutic drugs such as irinotecan (CPT-11) has improved the response rate, resistance usually develops, making chemotherapy ineffective (73,75,76). The mechanisms of resistance to chemotherapeutic drugs are usually associated with drug influx/efflux, drug activation/ inactivation, target protein alteration and DNA damage response (DDR) signaling (77-79). For example, it has been

reported that the expression of multidrug resistance protein (MRP) confers resistance to 5-FU in cancer cells by transporting the metabolites of 5-FU out of cells (80). High levels of 5-FU catabolic enzyme dihydropyrimidine dehydrogenase (DPD) in colorectal tumors are associated with resistance to 5-FU (81). CRC patients with high protein expression level of 5-FU target enzyme TS had a lower responsive rate toward 5-FU-based therapy (82). In addition, it has been demonstrated that CRC patients with DNA mismatch repair-defective tumors lacked response toward 5-FU-based therapy, and p53-deficient tumor, which was unable to activate p53-dependant apoptosis upon DNA damage, were resistant to 5-FU treatment (83). Similar mechanisms of resistance are also found in CPT-11-resistant tumors. For instance, MRP expression is also linked to CPT-11 resistance in CRC and other cancers (84-86). Furthermore, a clear relationship between chemosensitivity and the level of intracellular carboxylesterase, an enzyme required to convert CPT-11 to the more active form SN-38, has been demonstrated: cells with low carboxylesterase expression were more resistant to CPT-11 exposure (87,88). These studies suggest that a thorough understanding of the mechanisms of drug action is necessary for treatment design and patient stratification in cancer therapy.

1.2.3 Targeted Therapy Drug Resistance

Unlike traditional chemotherapy which attacks all rapidly growing cells, targeted therapy drugs specifically block the growth of tumors by interfering with the molecules needed for carcinogenesis and tumor growth. With the benefit of fewer side effects and more rational therapy design, targeted therapy provides a new avenue of cancer therapy. However, the benefits are at best transitory and are invariably followed by restoration of tumor growth due to the *de novo* and acquired resistance. Most targeted therapies are either small molecule drugs or

monoclonal antibodies. A common resistance mechanism for the small-molecule targeted agents is the secondary mutation in the kinase domain, which affects gatekeeper amino acids that control the access to a large hydrophobic pocket in the kinase where most inhibitors bind (89). For therapies using monoclonal antibodies, the activation of downstream effectors usually renders cancer cells resistant to the treatment. For example, cetuximab and panitumumab block the activation of EGFR and its downstream signaling including RAS-RAF-mitogen-activated protein kinase (MAPK) and PI3K-Akt cascades. It has been reported that mutations in *PIK3CA*, *KRAS*, and *BRAF*, and the loss of PTEN expression impair the response of mCRC to cetuximab and panitumumab (90-94). *KRAS* mutation analysis prior to anti-EGFR therapy has been incorporated into mCRC treatment guideline (95). Another resistance mechanism for monoclonal antibody therapy is the activation of alternative pathways. A subpopulation of mCRC patients refractory to cetuximab or panitumumab demonstrated HER-2 expression and were sensitive to trastuzumab (anti-HER-2 antibody) and lapatinib (HER-2 inhibitor) treatment (96). In addition, elevated hepatocyte growth factor receptor (HGFR) expression resulting from *MET* proto-oncogene amplification was also found in a subset of mCRC patients receiving anti-EGFR therapy (97). These studies underscore the importance of signaling crosstalk in the development of targeted therapy resistance and provide the molecular basis for combinatorial therapy using targeted agents.

1.2.4 Stress Response in the Chemoresistance

1.2.4.1 Overview

Cellular adaptive response is a multifaceted program generated in cells to cope with sublethal stresses originating from fluctuations in external conditions, including temperature,

oxygen tension, redox potentials, extracellular signals, and chemical insults such as chemotherapeutic drugs (98). During the adaptive process, cells undergo dramatic changes to adapt their metabolism and protect themselves from potential damages. This is accomplished through a concerted action of diverse stress response pathways including autophagy, ER stress signaling and senescence (98). There is accumulating evidence that reveals these adaptive responses are crucial for the survival of tumors following chemotherapy. For example, autophagy, a dynamic process in which double-membrane vesicles are formed to sequester cytoplasm or organelles, promotes tumor cell survival under anti-angiogenic therapy. Autophagy enhances cell survival by clearing damaged organelles, reducing cells' oxidative metabolism and providing nutrition when blood perfusion is limited (99,100). Treatment with EGFR-blocking antibody cetuximab is also known to induce autophagy in lung cancer cell lines to promote cell survival (101,102). ER stress signaling, on the other hand, promotes resistance by inducing the expression of pro-survival proteins. For instance, Wroblewski et al. showed that the treatment of BH3 mimetics Obatoclax and ABT-737 induces anti-apoptotic Bcl-2 family proteins Mcl-2 via ER stress signaling in melanoma (103). Finally, with a profound change in the secretome, senescent cells were demonstrated to secrete inflammatory cytokines, protease and growth factors to promote cell survival under stress conditions (104,105). Taken together, these findings suggest a complex network behind the adaptive response of cancer cells subjected to chemotherapy. More studies are needed to understand these mechanisms in order to enhance the anti-tumor efficacy of chemotherapy.

1.2.4.2 ER Stress signaling

Endoplasmic reticulum (ER) is a network of branching tubules and flattened sacs that control the synthesis, folding and modification of over a third of all cellular proteins. To optimally process protein synthesis and maturation, the functions of this protein-folding machinery are highly regulated to maintain ER homeostasis for cell survival (106). Conditions such as hypoxia, nutrient deprivation, oxidative insult and loss of calcium homeostasis can impair glycosylation or protein disulfide bond formation, causing accumulation of unfolded proteins in the ER and trigger an evolutionarily conserved protein quality control mechanism termed unfolded protein response (UPR) or ER stress signaling pathways (107,108). Activation of UPR induces two temporally distinct cellular activities to attenuate protein misfolding: An initial response to decrease protein synthesis and promote degradation of misfolded proteins, and subsequent transcriptional upregulation of target genes that may eventually trigger cell death in the cells beyond repair (106).

In the mammalian system, UPR comprises signaling pathways initiated by three transmembrane stress sensors located in the ER known as protein kinase RNA-like ER kinase (PERK), inositol-requiring kinase 1 α (IRE1 α) and activating transcription factor 6 (ATF6). In cellular homeostasis, GRP78 binds to the ER stress sensors, maintaining them in an inactive state. Under conditions of ER stress, GRP78 is titrated away by unfolded proteins to release stress sensors (107,109). Upon release, both PERK and IRE1 α undergo homo-oligomerization and autophosphorylation to activate their kinase activity. Activated PERK inhibits general protein translation through the phosphorylation of eukaryotic translation initiator factor-2 α (eIF2 α) at Ser 51 (110). Activated IRE1 α , on the other hand, initiates the splicing of X-box binding protein 1 (XBP-1) with its RNase domain, resulting in a potent and stable transcription

factor that controls the expression of genes encoding factors that modulate protein folding, secretion and lipid synthesis (111). After the dissociation from GRP78, ATF6 translocates to the Golgi apparatus and releases its cytosolic domain (ATF6f) through proteolytic cleavage. The fragmented ATF6 can act as a transcription factor to control the upregulation of a select set of UPR genes (112) (Fig. 1.2).

When stress persists and cellular homeostasis cannot be restored, the apoptotic signaling pathway is initiated and damaged cells are eliminated. CHOP (CCAAT/enhancer-binding protein homologous protein) is a nuclear transcription factor that inhibits expression of the gene encoding anti-apoptotic BCL-2, in addition to enhancing the expression of pro-apoptotic BCL-2 family members (113). CHOP is upregulated during the activation of ER-stress-associated cell death. For maximal induction of CHOP, the activation of all three ER stress signaling are needed, but the PERK/eIF2 α signaling plays a crucial role and is dominant over the other two pathways (114,115).

Studies have suggested that different UPR sensors may respond differentially to stress stimuli, and it is still not clear how the threshold of stress is determined for the sensors to shift their signaling output from promoting survival to cell death (116,117). The cell fate decision under stress is most likely achieved through the crosstalk between all the stress signaling and in a context-dependent manner.

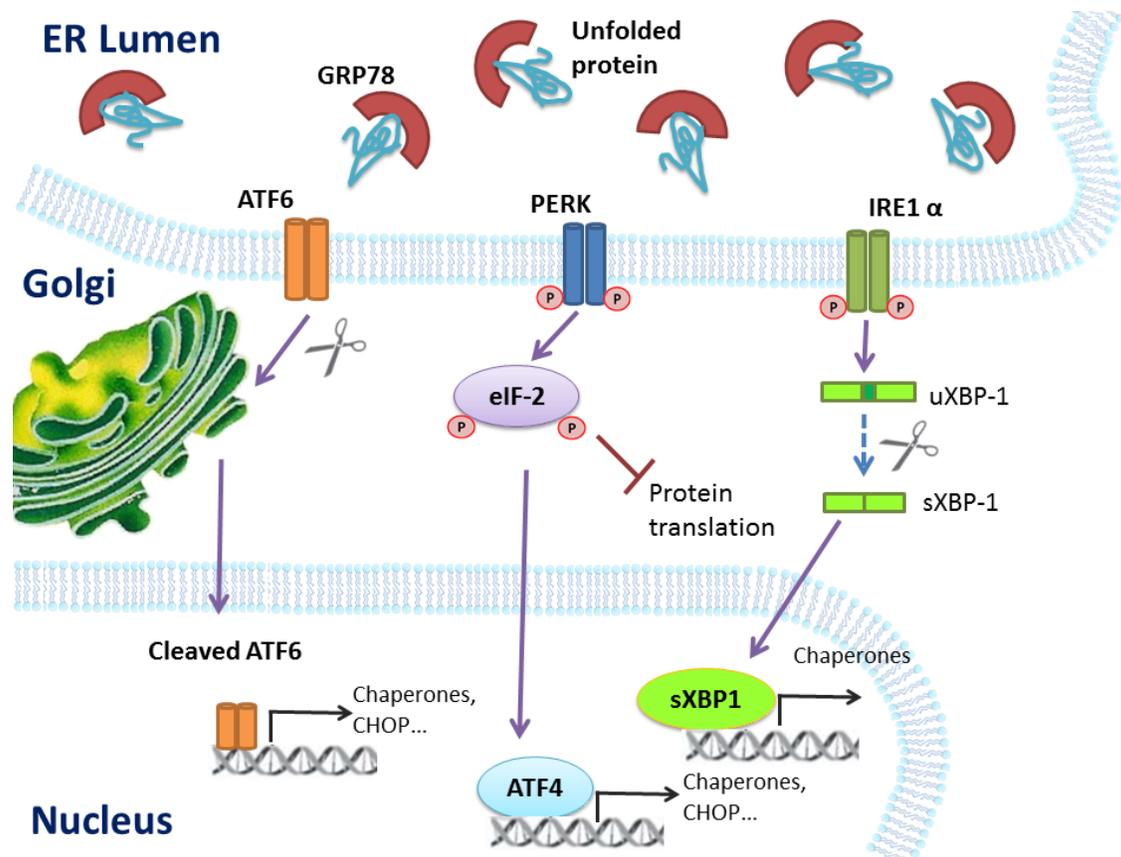


Figure 1.3. ER stress signaling activation. When cells are under ER stress, GRP78 dissociates from three ER stress sensors to bind the unfolded proteins. This results in the activation of the three ER stress sensors inositol-requiring kinase 1 α (IRE1 α), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6): IRE1 α activates its RNase activity to convert the unspliced mRNA of X-box binding protein 1 (uXBP-1) into spliced form (sXBP-1), which generate a more potent transcriptional factor. Activated PERK phosphorylates eukaryotic translation initiator factor-2 α (eIF2 α), resulting in an attenuation of global protein synthesis and the synthesis of activating transcription factor 4 (ATF4). ATF6 translocates from the ER to Golgi apparatus, where it is cleaved into the active form. ATF4, sXBP-1 and the cleaved ATF6 act in concert to induce the transcription of genes mediating protein folding, degradation and ER stress-associated cell death.

1.3 SPARC

Secreted protein acidic and rich in cysteine (SPARC), also known as osteonectin or basement-membrane-40 (BM-40), is a calcium and collagen-binding matricellular glycoprotein (118). As a member of the family of matricellular proteins, which modulate cell-matrix interactions and cell functions without participating in the structural scaffold of the matrix, SPARC is known to modulate cell spreading and attachment (119), inhibit cell cycle progression (120), influence the secretion and activation of matrix metalloproteinases (MMPs) (121), and to modulate the activity of several growth factors including fibroblast growth factor (FGF) (122,123), vascular endothelial growth factor (VEGF) (124), platelet-derived growth factor (PDGF) (125), and transforming growth factor β (TGF- β) (126). Although traditionally regarded as a matricellular protein, SPARC might also function intracellularly exerting different biological activities. For example, the expression of SPARC has been detected in the nucleus of lens epithelial, embryonic chicken and urothelial cells, and may function as a component of nuclear matrix and inhibit DNA synthesis in the cells (127-129). SPARC is also found in the endoplasmic reticulum and possesses chaperone activity (130-132). These studies suggest a complex biological network involving SPARC and more research is needed to elucidate the biology of this protein.

The complexity of SPARC is also reflected by its various effects on cancer cells. SPARC functions as a chemosensitizer in certain cancers, such as CRC (133-135), hepatocellular carcinoma (136), pancreatic cancer (137) and osteosarcoma (138). SPARC has been demonstrated to suppress cell cycle progression in both ovarian carcinoma (139) and acute myeloid leukemia (140). Loss of SPARC is associated with an accumulation of reactive oxygen species and urothelial cell proliferation in bladder cancer (141). In CRC, apoptosis can be

induced in the presence of significantly lower concentrations of chemotherapy when SPARC is overexpressed (133), by potentiating the activation of the extrinsic cascade of apoptosis via its interaction with caspase 8, and subsequent involvement of the intrinsic cascade, via Bid (134,135).

1.4 GRP78

Glucose-regulated protein, 78kDa (GRP78), also referred to as Bip/HSPA5, is a well-characterized ER chaperone and also a master modulator of UPR. During cellular homeostasis, GRP78 binds to the three ER stress sensors: PERK, IRE1 α and ATF6. However, under conditions of ER stress, GRP78 is titrated away by unfolded proteins to activate UPR and reduce cellular stress (107,109). Activation of the UPR promotes cell survival by reducing protein influx into the ER, and the selective synthesis of the components of the protein folding (117). However, when ER stress persists and homeostasis cannot be restored, UPR triggers cell death in cells that are beyond repair.

The upregulation of GRP78 expression has been shown to be associated with the development of chemotherapy resistance. For example, GRP78 is overexpressed in patients with castration-resistant prostate cancer (142,143) and brain endothelial cells resistant to CPT-11 and etoposide (144). Conversely, inhibition of GRP78 re-sensitizes B-lineage acute lymphoblastic leukemia cells that were previously refractory to vincristine (145), and an inhibitor targeting GRP78's ATPase domain has also been shown to resensitize breast cancer stem cells by inducing β -catenin proteasome degradation (146). Recently, the use of HA15, a thiazole benzenesulfonamides compound specifically targeting GRP78, was able to overcome resistance

to BRAF inhibitor both *in vitro* and *in vivo* in melanoma via ER stress. (147). Therefore, a strategy that targets GRP78 may increase the efficacy of cancer treatment.

1.5 HSP47

Heat shock protein 47 (HSP47), encoded by the *SERPINH1* gene, is a 47-kDa collagen-binding heat shock protein, which belongs to the serine protease inhibitor (serpin) superfamily. HSP47 is the only known substrate-specific ER chaperone, which transiently binds to newly synthesized procollagens in the ER to facilitate their maturation and collagen secretion (148). HSP47 expression in cells and tissues has been shown to correlate closely with collagen expression, and the upregulation of HSP47 is associated with abnormal collagen deposition and fibrotic diseases (149). Recently HSP47 was identified to regulate ER stress signaling by facilitating IRE1 α oligomerization via direct interaction, suggesting HSP47 may possess various functions other than acting as a collagen chaperone in the cells (150).

However, the role of HSP47 in cancer is largely unexplored and the existing studies appear to be contradictory. HSP47 was reported to be upregulated in several cancers (151-154) and the increased HSP47 expression is associated with poor prognosis (155,156). Suppression of the HSP47 expression by short interfering RNA significantly suppressed cell proliferation, migration and invasion in cervical cancer (153). Moreover, the expression of HSP47 was found to be activated during breast cancer development and progression, and the silencing of HSP47 led to the inhibition of tumor growth *in vivo* (157). In contrast, in neuroblastoma, HSP47 expression was silenced in tumors and several cancer cell lines due to aberrant methylation of promoter CpG islands (158). The molecular mechanism by which HSP47 exerts its function in different cancers is still unclear although it most likely occurs in a context-dependent manner.

1.6 Thesis Theme and Rationale

The endoplasmic reticulum (ER) is an essential organelle that carries out fundamental cellular activities including protein production, calcium storage, redox regulation and drug metabolism (159). Therefore, the ability of cancer cells to survive and grow under therapeutic challenges is considerably dependent on the functional status of the ER. The deregulation of ER homeostasis and therefore the underlying ER proteins (ER proteome) has been observed in many cancers (160-162). This is reflected by alterations of ER protein expression levels, posttranslational modifications, or abnormal localization of ER-resident proteins in cancer cells (163).

In previous studies from our laboratory, SPARC was identified to be downregulated in chemoresistant CRC cells, and its expression increased the sensitivity of CRC cells exposed to chemotherapy (133,134). Although traditionally regarded as a matricellular protein, intracellular immunostaining of SPARC has been reported in various tissues, and the functions of intracellular SPARC are gradually emerging (129,132,164). For example, we previously found that SPARC enhances apoptosis via its interaction with pro-caspase-8 in CRC cells (134). To further understand the mechanisms by which SPARC exerts its chemosensitizing functions in CRC cells, we conducted immunoprecipitation followed by tandem mass spectrometry (MS/MS) analysis to identify potential SPARC-interacting partners. We found GRP78, an ER stress modulator and a promoter of chemoresistance in various cancers, as a putative binding protein of SPARC (165,166). Therefore, the first section of this thesis focuses on exploring the molecular mechanism by which the GRP78-SPARC interaction influences the response of CRC cells under chemotherapy-induced stress.

Another SPARC-binding protein identified is another ER-resident stress protein HSP47. Like SPARC, HSP47 is also an evolutionary conserved collagen-binding protein, but its role in cancer remains largely unexplored. As recent studies revealed, many ER-resident proteins are overexpressed in tumors and perform atypical functions distinct from their normal physiological roles (107). We were curious to understand whether HSP47 is also crucial, like SPARC and GRP78, in mediating a more sensitive chemotherapeutic response in CRC. Hence, in the second section of this thesis, we investigated HSP47's role in CRC, especially focusing on HSP47's effects on the chemotherapeutic response and its underlying molecular mechanism.

1.7 Hypotheses and Specific Aims

The hypotheses and the specific aims for the two sections of this thesis are stated as below:

Chapter 3

Hypothesis:

SPARC suppresses tumor survival following exposure to chemotherapy in part through its interference with ER stress response and its interaction with GRP78

Specific aims:

1. Examine the protein interaction between SPARC and GRP78 under ER stress in colorectal cancer cells exposed to chemotherapeutic drugs
2. Study the effects of SPARC on ER stress signaling and the molecular mechanism involved in colorectal cancer cells following chemotherapy

3. Examine the clinical relevance of the relative expression level of GRP78 and SPARC in colorectal cancer cells.

Chapter 4

Hypothesis:

HSP47 modulates the chemotherapeutic response by promoting survival of colorectal cancer cells.

Specific aims:

1. Investigate the role of HSP47 in the chemotherapeutic response of colorectal cancer cells.
2. Explore the molecular mechanisms in which HSP47 is involved in modulating chemoresistance in colorectal cancer *in vitro*.
3. Examine the effects of HSP47 expression in colorectal cancer cells following chemotherapy *in vivo*.

Chapter 2: Materials and Methods

Common materials and methods used in both Chapter 3 and Chapter 4 are described in Chapter 2. Material and methods specific to each study were described after the introduction in the respective chapter.

2.1 Parental and Resistant Cell Lines

Human CRC cell lines RKO, HCT116 and CCL228 were purchased from American Type Culture Collection (ATCC) (Manassas, VA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, CA) supplemented with 10% newborn calf serum (NCS) at 37°C in a humidified atmosphere with 5% CO₂. The poorly differentiated human colorectal cancer cell line MIP101 overexpressing SPARC (MIP/SP) or empty vector pcDNA3.1 (MIP/Zeo) were maintained in DMEM with 10% NCS and 0.1% Zeocin (Invitrogen) (133,167). Human CRC cell line HT29 was purchased from ATCC and maintained in DMEM with 10% fetal bovine serum (FBS) (Invitrogen). Resistant cells MIP/5FU, RKO/5FU, RKO/CPT, HCT116/5FU and HCT116/CPT were maintained with 200 µM 5-FU, 100 µM 5-FU, 70 µM CPT-11, 1.25 µM 5-FU, and 1.25 µM CPT-11, respectively.

2.2 Immunoblotting

Cells were extracted in lysis buffer [1% Triton-X 100, 120mM NaCl, 50mM Tris-HCl, pH7.5] supplemented with 1% proteinase inhibitor cocktail (Sigma) followed by protein quantification with Bradford assay (Bio-Rad). An equal amount of proteins was separated by SDS-PAGE under reducing conditions and electrotransferred onto a PVDF membrane (Millipore). Blots were incubated with primary antibodies in TBST (TBS containing 0.1%

Tween-20) overnight at 4°C. The blots were then incubated with anti-mouse immunoglobulin (IgG)-HRP (SH023, ABM) or anti-rabbit IgG-HRP (cat.7074, CST) followed by enhanced chemiluminescence (ECL) detection. For immunoprecipitation analysis, conformation-specific secondary antibodies (ab131366, Abcam) were used.

2.3 Immunofluorescence Analysis

Cells were fixed with 4% paraformaldehyde at room temperature (RT) for 15min followed by incubation with blocking buffer (PBS with 5% NCS, 0.2% Triton X-100) for 1h at RT and primary antibodies at 4°C overnight. The proteins were detected with Alexa 488- Goat anti-Mouse IgG, Alexa 647 Goat anti-Mouse IgG or Alexa 555- Goat anti-Rabbit IgG (Invitrogen) for 1h at RT, followed by nucleus staining with Hoechst33258. For the tumor tissues staining, 5- μ m slices of the FFPE tissues sections were deparaffinized in xylene and rehydrated through graded ethanol. The sections were incubated with citrate buffer (pH 6.0) in 95°C water bath for 20 min for antigen retrieval followed by regular immunofluorescence staining described above.

2.4 Q-RT-PCR

Total RNA was extracted with Trizol (Invitrogen) according to the manufacturer's instruction. Oligo(dT)-primed first-strand cDNA was synthesized from 1 μ g RNA by using Reverted H Minus Reverse Transcriptase (Thermo) following the manufacturer's instruction. The transcript levels of RPL19 were used for normalization. qRT-PCR was performed by using KAPA SYBR FAST qPCR kit (KAPA Biosystems) in ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The following primers were used for qRT-PCR: RPL19: 5'-TGAAATCGCCAATGCCAACTC-3' (sense) and 5'-GGCTGTACCCTTCCGCTTACC-3'

(antisense); GRP78: 5'-GGCCGAGGAGGAGGACAAGA-3' (sense) and 5'-GGCGGCATCGCCAATCAGAC-3' (antisense); SPARC: 5'-CCCTGTACTGGCAGTTCG-3' (sense) and 5'-CCAGGGCGATGTACTTGTCA-3' (antisense). HSP47: 5'-CTGCAGTCCATCAACGAGTG-3' (sense) and 5'-CAGCTTTTCCTTCTCGTCGT-3' (antisense). The transcript levels of RPL19 were used for normalization.

2.5 MTS Cell Viability Assay

Cells were seeded at a density of 2000~ 4000 per well in 96-well plates. After 24 h, cells were treated with drugs of various concentration for 48h. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay using a test kit (Promega, WI, USA) was performed according to the manufacturer's instruction.

2.6 TUNEL Assay

The suspension and attached cells were harvested following drug treatment, and fixed unto glass slides with Shandon cytospin at 2000 rpm for 10 min, and stained with In Situ Cell Death Detection kit (Promega). The number of TUNEL-positive cells was counted and averaged from four independent experiments.

Chapter 3: The Interaction between SPARC and GRP78 Interferes with ER Stress Signaling and Potentiates Apoptosis via PERK/eIF2 α and IRE1 α /XBP-1 in Colorectal Cancer

3.1 Introduction

Although the overall mortality rate of CRC has been declining in the past few years, the survival rate for metastatic CRC patients remains low in part due to cancer cells' resistance to chemotherapy. By taking genome-wide approaches, our laboratory previously identified SPARC to be differentially downregulated in several resistant CRC cell lines (133). Importantly, the reexpression of SPARC was able to restore the sensitivity of xenografted tumors originally refractory to 5-FU treatment (133), suggesting that modulation of SPARC expression may influence CRC's chemosensitivity, and SPARC-based therapy may improve the treatment efficacy. Further studies on SPARC in our lab confirmed its chemosensitizing effects. For example, we found that SPARC promoter is hypermethylated in CRC and treatment with demethylating agents increased SPARC expression and improved therapy response (168). We also discovered that SPARC may enhance chemotherapy by augmenting apoptosis signaling via its interaction with procaspase 8 (134). To further understand the mechanisms by which SPARC promotes chemosensitivity in CRC, we conducted immunoprecipitation-mass spectrometry analysis and identified GRP78 and HSP47 as two promising SPARC-binding partners. Studies in this chapter will focus on how the interaction between SPARC and GRP78 influences the chemotherapeutic response of CRC cells. I first validated the binding and co-localization of these two proteins by immunoprecipitation and immunofluorescence assays. As I noticed that the

GRP78-SPARC interaction changed in response to ER stress induced by chemotherapy, I further examined the ER stress signaling in SPARC-overexpressing cells following chemotherapy. In addition, the relative expression level of GRP78 to SPARC was also observed to be associated with the chemosensitivity of CRC cells and the prognosis of CRC patients. Overall, I demonstrate in this chapter a detailed mechanism by which SPARC enhances the chemosensitivity of CRC cells by promoting ER stress-associated death via its interaction with GRP78 in CRC cells exposed to chemotherapeutic drugs.

3.2 Materials and Methods

3.2.1 Mass Spectrometry Analysis

A C-terminal V5 epitope-tagged SPARC cDNA vector was transiently expressed in MIP101 cells. SPARC protein complexes were immunoprecipitated with the anti-V5 antibody (Applied Biomaterials Inc) and eluted with elution buffer (V5 peptide 400ug/ml, ammonium bicarbonate 50 mM). Proteins immunoprecipitated with an empty vector transfection served as controls. The samples were run on 4-12% precast NuPAGE gels (Invitrogen) and each lane was cut into 16 horizontal slices. The gel slices were processed for tandem mass spectrometry analysis using in-gel dehydration, alkylation, trypsin digestion, and extraction (169). The peptides were analyzed by HPLC-electrospray-tandem mass spectroscopy (ESI-MS/MS) on a 4000 QTrap mass spectrometer (Applied Biosystems/Sciex, Foster City, CA, USA) using standard procedures (169). The MS/MS spectra emanating from the gel slices for each lane were concatenated and searched against the UniProt human database using the X!Tandem (<http://www.thegpm.org/tandem>) and Mascot (Matrix Science, Boston, MA, USA) search

algorithms. Candidate interacting proteins were those that were observed with two or more peptides and not in the control sample.

3.2.2 RT-PCR Assay for XBP-1 Splicing Detection

Total RNA preparation and cDNA synthesis were processed as described in section 2.4, followed by PCR with Platinum® Taq DNA Polymerase High Fidelity (Invitrogen) using primers flanking the splice site (170). The primer sequences for sXBP-1 detection were: XBP1: 5'-TTACGAGAGAAAACATCATGGCC-3' (sense) and 5'-GGGTCCAAGTTGTCCAGAATGC-3' (antisense); β -actin: GCCACGGCTGCTTCC-3' (sense) and 5'-GGCGTACAGGTCTTTGC-3' (antisense). The PCR condition was 94°C for 2 minutes, followed by 94°C for 30 seconds, 58°C for 30 seconds and 68°C for 30 seconds for 27 cycles. Unspliced XBP-1 gave a product of 289bp and the spliced cDNA of 263bp. PCR products were separated by 5% urea denaturing PAGE followed by ethidium bromide staining and quantification by ImageJ (National Institute of Health, USA).

3.2.3 Cell Fractionation and Immunoprecipitation

MIP/SP and HCT116 cells were seeded into 100mm dish overnight. MIP/SP cells were lysed in lysis buffer [25mM Tris-HCl (pH7.4), 150mM NaCl, 1% NP-40 and 5% Glycerol]. HCT116 cells were subjected to modified cell fractionation protocol(171) after treatment with 5-FU or tunicamycin (TM) (cat. 654380, Millipore). The treatment durations with TM and 5FU were different, as these were based on preliminary experiments that indicated that TM induction of ER stress occurred at earlier time points than 5-FU. HCT116 cells were lysed in Buffer A [10mM HEPES-KOH (pH7.9), 1.5mM MgCl₂, 120mM KCl and 0.2mM PMSF] and incubated

for 10min. Samples were centrifuged at 14,000 rpm for 1min to collect the cytosolic fraction. The cell pellets were resuspended in Buffer C [20mM HEPES-KOH (pH7.9), 25% Glycerol, 420mM NaCl, 1.5mM MgCl₂, 0.2mM EDTA and 0.2mM PMSF] and incubated for 20min. After centrifugation at 14,000 rpm for 2min, the supernatant was collected as nuclear fractions and the pellets were resuspended in IP lysis buffer and incubated for 30min. Samples were centrifuged 14,000 rpm for 10min and the supernatant containing membrane fractions were collected. The experiments were conducted at 4°C and all buffers were supplemented with 1% proteinase inhibitor cocktail (Sigma). 1mg of MIP/SP cell lysate was incubated with 5µg mouse anti-human SPARC antibodies (HTI, AON-5031), rabbit anti-human GRP78 antibody (Santa Cruz, sc-13968) or rabbit anti-PERK antibody (Santa Cruz, sc-13073) at 4°C overnight, and immunoprecipitated with Sepharose-Protein G beads (Sigma, P3296) or anti-Rabbit IgG beads (Rockland, 00-8800-25), respectively. Seven hundred micrograms of proteins from each subcellular fraction were used for immunoprecipitation following the same experimental conditions. After washing with IP lysis buffer, the beads were boiled in Laemmli buffer and centrifuged at 14,000 rpm for 1 min. The proteins were resolved by SDS-PAGE (12% gel) and subjected to Western blot analysis as described below.

3.2.4 RNA Interference

HCT116 cells were plated in 6-well plate at 80% confluency. After 24h, cells were transfected with iLenti-GFP SPARC siRNA or the scramble control siRNA (ABM) using Lipofectamine 2000 (Invitrogen). Cells were selected with puromycin (1µg/ml).

3.2.5 Colocalization Analysis

Images were captured on a Leica TCS SP5 II confocal microscope (Leica Microsystems) with 100x oil objective lenses and numeric aperture of 1.40N. Images of the cells were acquired from a 0.13- μm optical section, and no labeling was observed when using the secondary antibody alone. Each 3D stack image was deconvolved using Huygens Professional software (version 15.05) (Scientific Volume Imaging) followed by colocalization analysis. Quantitative assessment of colocalization between SPARC and GRP78 was performed by the JACoP plugin in ImageJ (NIH) as described (172). ImageJ colocalization plugin was used to identify and highlight the colocalizing pixels in white. Colocalization Finder plugin was also used to highlight the colocalizing pixels of SPARC and GRP78 on ER-tracker staining image. The percentage of colocalizing signals on ER was calculated based on the following formula: (Area of the highlighted regions in which the average ER tracker staining intensity is above the selected threshold)/ (Total area of the highlighted regions) x 100 %. Intensity threshold was selected to minimize the nuclear regions while including the majority of the ER regions.

3.2.6 Tissue Microarray (TMA) construction and Immunohistochemistry (IHC)

A TMA representing 143 surgically resected colorectal neoplasias, was constructed by obtaining two formalin fixed, paraffin-embedded cores from representative areas of primary tumors from each patient. 4 μm -thick sections from the TMA block were deparaffinized in xylene and rehydrated. Sections were then heated in citrate buffer for 15 minutes for antigen retrieval. Endogenous peroxidase activity was blocked using 0.3% H_2O_2 and washed with PBS for 10 minutes. Immunohistochemical staining with primary antibody against SPARC was carried out using Ultravision LP detection kit (Thermo Fisher Scientific, Fremont, CA, USA).

Sections were treated with Ultra V Block for 5 minutes to prevent nonspecific reaction with primary antibodies, then incubated at 4°C for 24 hours with primary antibodies, followed by incubation with a primary antibody enhancer for 10 minutes at room temperature. Subsequently, sections were treated with HRP polymer for 15 minutes and the reaction product was developed using 3,3-diaminobenzidine tetrahydrochloride (Zymed, South San Francisco, CA, USA). The sections were counterstained with hematoxylin and mounted with Tissue-Tek Glas 6419 (Sakura Finetek, Torrance, CA, USA). Negative controls consisted of omission of the primary antibodies. Staining expression scores were based on the number of tumor cells with positive staining in the cytoplasm, and were categorized as follow: 0 or none (expression <10%), 1+ or weak (10-50%), 2+ or strong (50-80%), and 3+ or intense (>80%), by two independent pathologists who were blinded to clinicopathological data. The two expression scores per sample were averaged, with the average representing the patient's final expression intensity.

3.2.7 Statistics

Kaplan-Meier method and the Cox regression model were used for univariate survival analysis. Kaplan-Meier Method was used to estimate the survival functions, and median survival times and their 95% confidence intervals. The hazard ratios and their 95% confidence intervals were obtained using Cox regression. The independent variables included the staining intensity of GRP78 and SPARC. Conditional inference trees were used to group those ordinal variables as binary variables. Independent variables were then correlated to overall survival and distant relapse-free survival.

3.3 Results

3.3.1 A Novel Interaction between SPARC and GRP78 Occurs in CRC

In order to help further understand the mechanisms by which SPARC may facilitate apoptosis in response to chemotherapy, we began by identifying potential SPARC-interacting partners. We used C-terminal V5 epitope-tagged SPARC expressed in MIP101 cells to co-immunoprecipitate (co-IP) SPARC-interacting protein complexes. Following co-IP, the complexes were digested with trypsin and subsequently processed for tandem mass spectrometry (MS/MS) analysis. To exclude non-specific binding proteins, empty vector-transfected MIP/ZEO cells were used as co-IP control. Glucose-regulated protein 78 kDa (GRP78) was identified with high peptide coverage as a putative SPARC interacting protein (Table 3.1).

In order to confirm the interaction between GRP78 and SPARC, co-IP analysis using intrinsically high SPARC-expressing human CRC cell line HCT116 and SPARC-overexpressing MIP/SP were conducted. The results confirmed the presence of an interaction between GRP78 and SPARC in endogenous SPARC-expressing HCT116 (Fig. 3.1.A) and SPARC-overexpressing MIP/SP but not MIP/Zeo cells. (Fig. 3.1.B). However, the immunoblotting results of the co-IP assay using endogenous HCT116 cells show a weak signal, suggesting a low-affinity interaction between GRP78 and SPARC. Densitometry analysis further indicates a large protein stoichiometry ratio between these two proteins, suggesting that these two proteins perhaps interact indirectly through a protein complex. Further studies are needed to characterize this interaction.

In addition, confocal microscopic immunofluorescent analysis also suggested colocalization of GRP78 (Fig. 3.1.C-i) with SPARC (Fig. 3.1.1C-ii) in HCT116. Given GRP78's role as an ER-chaperone, it was interesting to note that the majority of the colocalization pixels

demonstrating the interaction between GRP78 and SPARC overlapped with the ER tracker-labeled regions ($65.4 \pm 4.5\%$, N=3) (Fig. 3.1.C-iii), suggesting that the interaction between GRP78 and SPARC may be occurring predominantly in the ER (Fig. 3.1.C-iv, v).

Table 3.1. Immunoprecipitation- Mass Spectrometry Identification of SPARC Interacting Proteins.

The MS identification scores for SPARC and candidate interacting proteins are shown.

Bait protein	X!Tandem			Mascot			Accession
	Log(E)	#	%	Score	#	%	UniPROT
	^a	peptides	coverage	^b	peptides	coverage	
SPARC Secreted protein acidic and rich in cysteine (C-term V5 epitope tag)	-56	8	22	310	11	33	P09486
Interacting protein							
GRP78 78 kDa glucose-regulated protein	-86	11	25	484	22	39	P11021

^a – The log(E) value is an estimate of the probability that the protein assignment occurred randomly, as calculated by the X!Tandem algorithm against the human UniPROT protein database.

^b – Mascot score, as calculated by the Mascot algorithm against the human UniPROT protein database

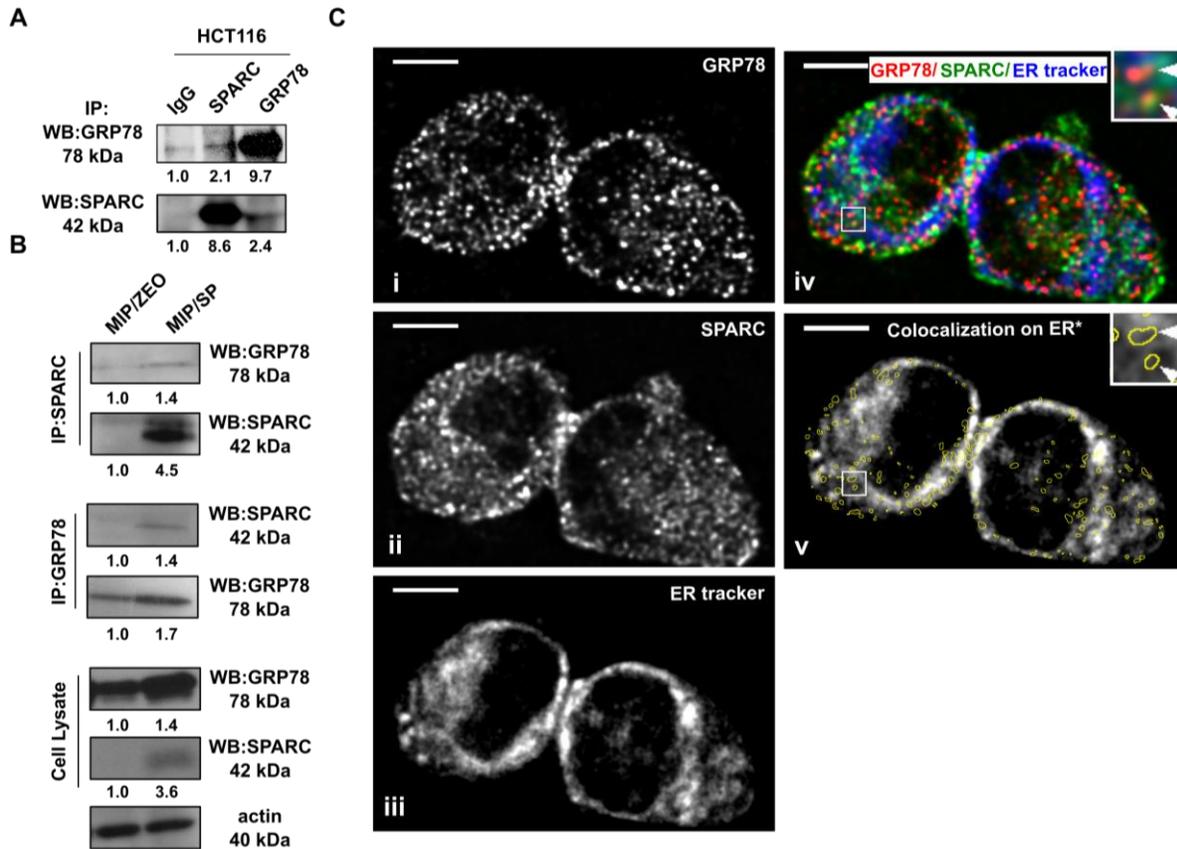


Figure 3.1. Interaction between GRP78 and SPARC in CRC. Co-immunoprecipitation of SPARC with GRP78 in (A) HCT116 and confirmed in (B) MIP/Zeo and MIP/SP cells. (C) Representative images of the colocalization between (i) GRP78 and (ii) SPARC in HCT116 by confocal microscopy and immunofluorescent analysis. (iii) Endoplasmic reticulum was stained with ER tracker blue-white DPX dye. (iv) Images were overlapped, and (v) the GRP78:SPARC colocalization pixels were identified by Colocalization Finder plugin in ImageJ and highlighted with yellow circles. The average ER tracker staining intensity in the highlighted areas was calculated to determine the percentage of GRP78:SPARC colocalization in the ER. (N=3) Scale bar= 5 μ m.

3.3.2 A Dynamic Interaction between SPARC and GRP78 in the ER Occurs in the Presence of ER Stress

Based on the confocal microscopy findings suggesting that the interaction between GRP78 and SPARC occurs predominantly in the ER, we proceeded to specifically examine this possibility by co-IP/immunoblot analysis. Given GRP78's known ER-stress modulatory function, we were also interested in examining whether the interaction between GRP78/SPARC could be influenced by the level of ER stress in CRC cells. Co-IP assays were performed following cellular fractionation of endogenous-SPARC expressing HCT116 cells that were treated with either 25 μ M 5-FU or 1 μ g/ml TM. The interaction between endogenous GRP78 and SPARC was mainly observed in the ER-containing microsomal fractions (Fig 3.2.A, similar fraction that contained ER-specific calnexin). Interestingly, this interaction increased following ER-stress induced by 5FU and TM, predominantly within the microsomal fraction, and to a lesser extent, in the nuclear fraction (Fig. 3.2.A, DNA-PK positive fraction). We also characterized this interaction, using another chemotherapy, CPT-11, and noticed a similar increase in the interaction between GRP78 and SPARC in the microsomal fractions (Fig. 3.2.B). These observations lead us to believe that the interaction between SPARC and GRP78 is augmented in the setting of ER-stress.

The interaction that occurs between GRP78 and SPARC in response to ER stress was also further evaluated by confocal microscopy. In HCT116 cells, a greater concentration of colocalized pixels was seen following the induction of ER stress with CPT-11 exposure (Fig. 3.3.A, B and C). Similarly, MIP/SP cells exposed to ER stressor tunicamycin (TM) resulted in an increase in the interaction between these two proteins in the perinuclear region (as indicated by an increase in colocalized pixels, Fig. 3.4). Quantitative analysis demonstrated that following

CPT-11 treatment, a statistically larger percentage of SPARC and GRP78 signals overlapped as represented by higher Pearson's (by $R_r= 0.265\pm0.028$ vs. $R_r= 0.392\pm0.024$, $P<0.01$) and Mander's overlap coefficient ($R= 0.877\pm0.010$ vs. $R= 0.915\pm0.006$, $P<0.01$) (Fig. 3.3.C and D). This indicates that a dynamic interaction between GRP78 and SPARC exists that is dictated by changes in cellular stress (for example, following exposure to chemotherapy).

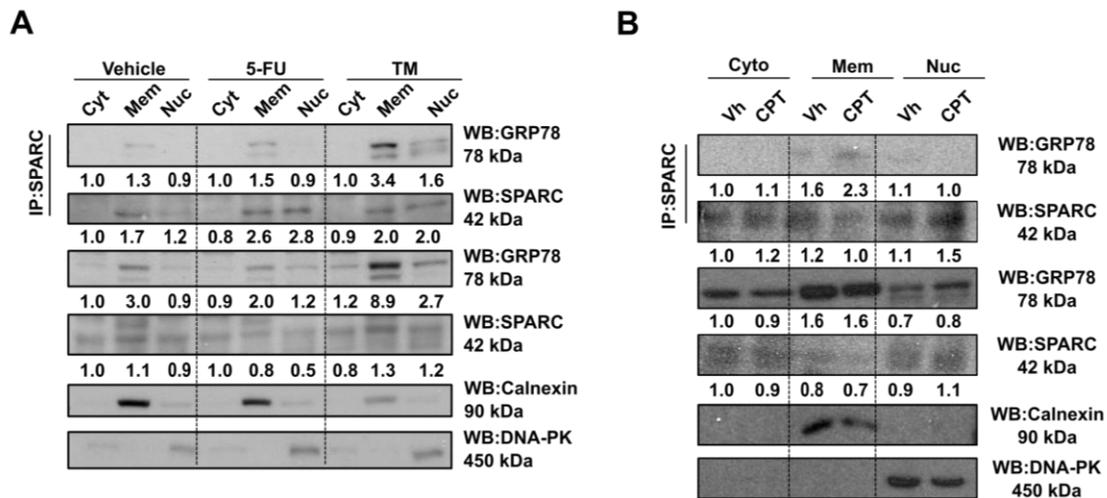


Figure 3.2. Increased interaction between GRP78 and SPARC in the membranous fractions of HCT116 cells under 5-FU- and CPT-11-induced ER stress. HCT116 were treated with 5-FU (25 μ M, 12h), TM (1 μ g/ml, 6h) or (B) CPT-11 (50 μ M, 12h) followed by cell fractionation, co-IP and Western blot analysis of cytoplasmic (Cyt), membranous (Mem) and nuclear (Nuc) fractions. Calnexin and DNA-PK served as quality control of cell fractionation. Experiments were repeated (N=3) and the representative images are presented.

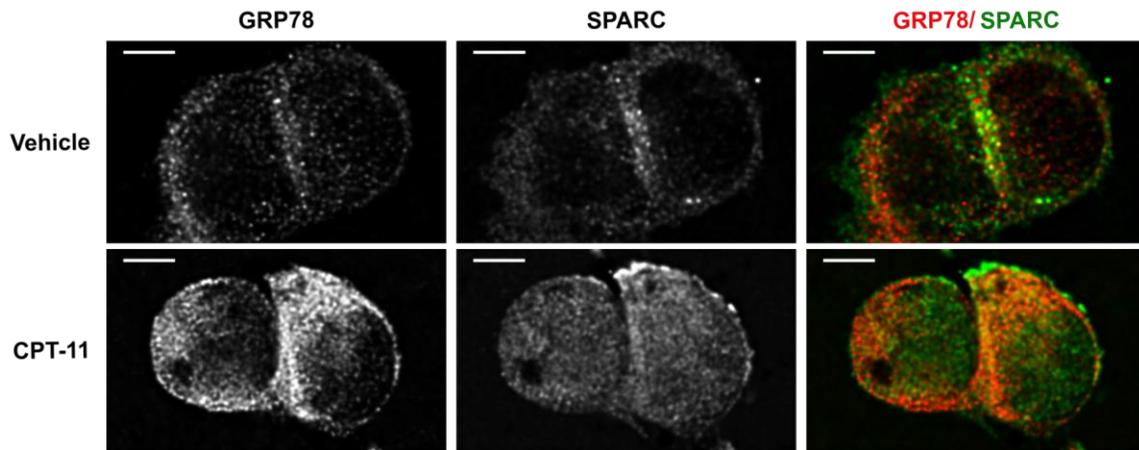
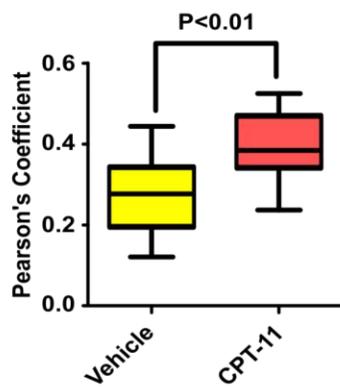
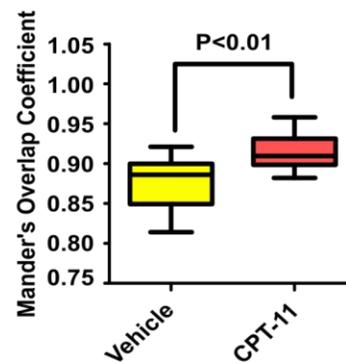
A**B****C**

Figure 3.3. Increased colocalization between GRP78 and SPARC in HCT116 cells under CPT-11 treatment. (A) Immunofluorescent analysis of HCT116 cells treated with CPT-11 (50 μ M, 12h) or vehicle by confocal microscopy. Cells were labeled with primary antibodies against SPARC (green) and GRP78 (red). Quantitative colocalization analysis was performed followed by deconvolution of the images. Scale bar= 5 μ m. (B) The Pearson's coefficient and (C) the overlap coefficient was calculated from twelve images (N=12) for each group.

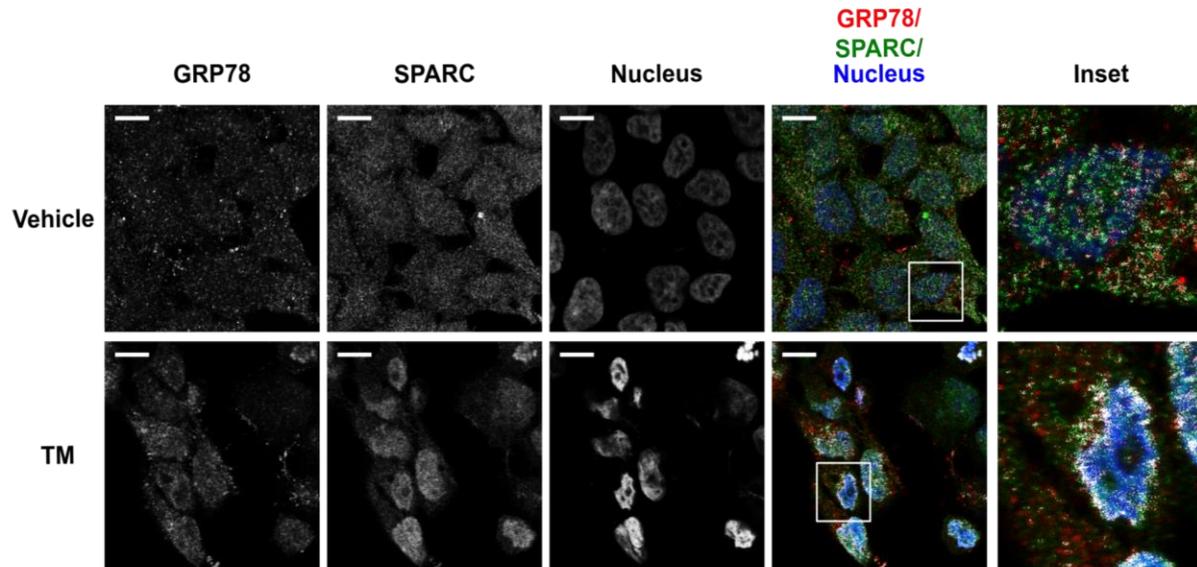


Figure 3.4. Colocalization between GRP78 and SPARC in the perinuclear region of SAPRC-overexpressing MIP/SP exposed to ER stress-inducer tunicamycin. Immunofluorescent analysis of MIP/SP cells treated with TM (1 μ g/ml, 6h) or vehicle. Colocalization of GRP78 and SPARC was evident in TM-treated cells based on the analysis using the ImageJ Colocalization Finder plugin (white). Fluorescence intensities were gamma-corrected (gamma= 2.0) to reveal the colocalization pixels in the perinuclear region. Scale bar= 10 μ m.

3.3.3 GRP78 Reduces SPARC's Pro-apoptotic Effects During ER Stress

Based on the above observations that the interaction between GRP78 and SPARC increased under conditions of cellular ER stress (as in the presence of chemotherapy), we wondered if changes in the expression level of the two proteins would not only alter their protein-interaction, but also influence cell viability following exposure to ER-stress, including chemotherapy. To answer this question, we modulated GRP78 expression level in the SPARC-overexpressing MIP/SP cells and evaluated the viability of MIP/SP and MIP/SP/78 cells (MIP101 cells co-expressing SPARC and GRP78) following exposure to TM and 5-FU. The overexpression of GRP78 in MIP/SP cells increased cell viability despite exposure to 5-FU and TM: cell viability increased from less than 50% in MIP/SP following 5-FU exposure to more than 60% in MIP/SP/78 cells. A similar trend was also observed following tunicamycin exposure (Fig 3.5.A). This is also represented by a reduction in TUNEL positive apoptotic MIP/SP/78 in comparison to MIP/SP following treatment of 5-FU and tunicamycin (Fig. 3.5.B). These results suggest that the expression of GRP78 may reduce SPARC's ability to promote ER stress-associated death in CRC cells following chemotherapy. Furthermore, it demonstrates that it is the relative expression level of GRP78 to SPARC that influences the survival of cancer cells in response to chemotherapy.

To confirm that the relative expression of GRP78:SPARC is a better determinant of cell viability in CRC, several CRC cell lines were examined, with the corresponding IC₅₀ values of 5-FU and CPT-11 determined by MTS assays. No correlation was observed between the GRP78 expression level (Fig. 3.6.C, left panel) and the IC₅₀ for 5-FU (Fig. 3.6.A) and CPT-11 (Fig. 3.6.B) in the CRC cells (5-FU: HT29, 373.31±61.44 μM; CCL227, 123.19±19.85 μM; HCT116,

73.02±15.20 μ M. CPT-11: CPT-11: HT29, 41.73±6.42 μ M; CCL227, 30.94±1.70 μ M; HCT116, 21.13±2.69 μ M).

However, SPARC expression level was found to be the lowest in the most resistant HT29, although its expression is higher in the CCL227 compared to the more sensitive HCT116 cells. (Fig. 3.6.C, *middle panel*). Again, we observed that the relative expression level of GRP78 to SPARC may be a better indicator of CRC cells' response toward chemotherapy: while the most resistant HT29 has the highest ratio of GRP78 to SPARC expression level, the most sensitive HCT116 also shows the lowest relative expression level among the three (Fig. 3.6.C, *right panel*). We also observed similar results in our MIP 101 and RKO 5-FU and CPT-11 resistant cell lines (Fig. 3.6.D and E). This interesting finding suggests that the ratio of GRP78 to SPARC expression may be a better indicator of a cell's ability to survive following exposure to chemotherapy, and consequently, a better predictor of resistance to therapy. This potential observation motivated us to examine whether the ratio of GRP78 to SPARC expression level could serve as a prognostic biomarker for patients with CRC (see below).

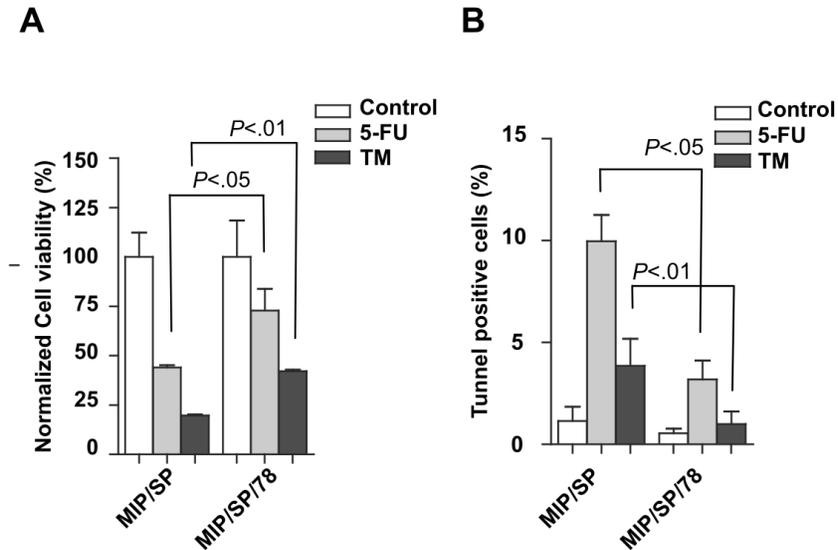


Figure 3.5. GRP78 overexpression increases the cell viability and suppresses apoptosis induction in SPARC-overexpressing MIP/SP under the treatment of 5-FU and ER stressor tunicamycin. The effects of SPARC and GRP78 expression on cell viability under ER stress were determined by using MIP/SP and GRP78-overexpressing MIP/SP cells (MIP/SP/78) following exposure to 5-FU (5 μ M) and TM (1 μ g/ml) for 48h. Cell viability and apoptosis were analyzed by (A) MTS and (B) TUNEL assays, respectively. Data represent mean \pm SEM, N=3.

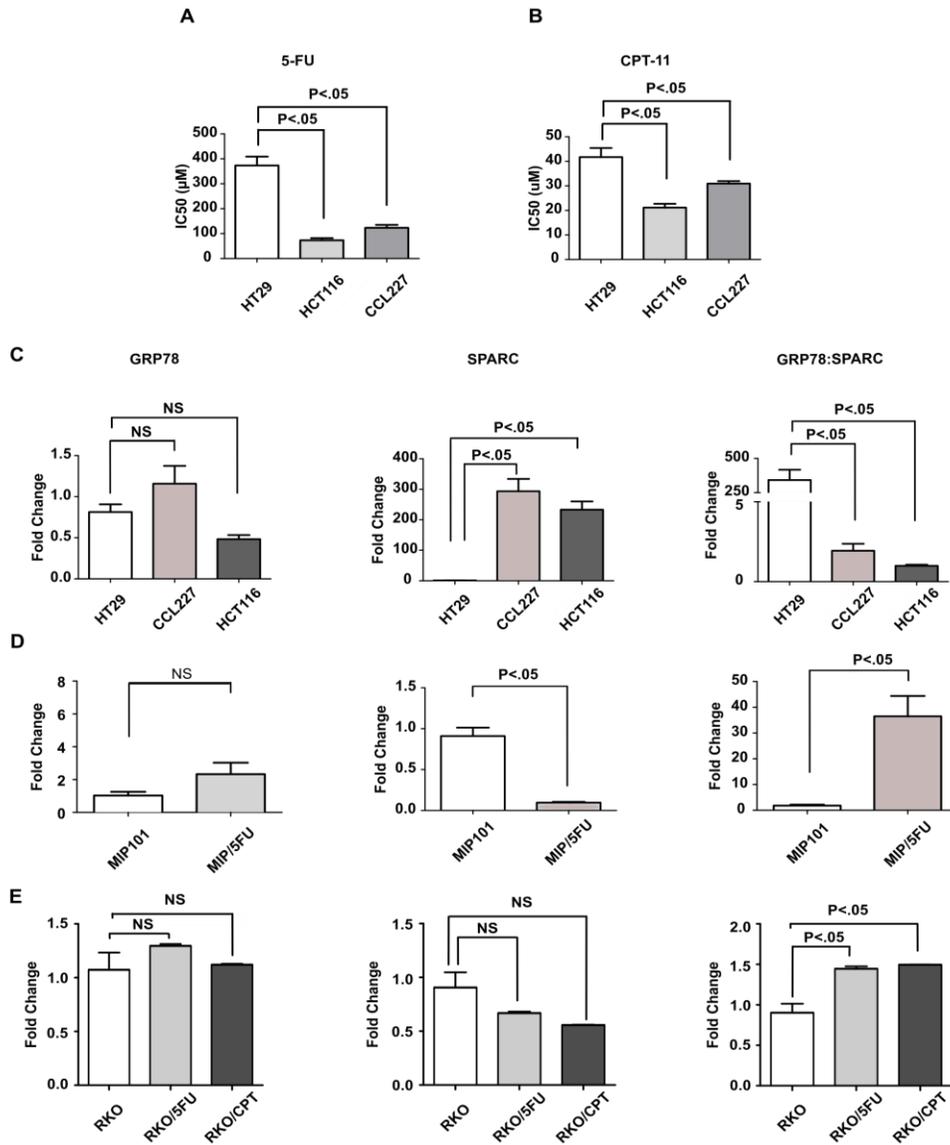


Figure 3.6. CRC cell lines with high relative expression level of GRP78 to SPARC are more resistant to drugs. Cell viability and IC₅₀ of CRC cell lines HT29, CCL227 and HCT116 following exposure to (A) 5-FU and (B) CPT-11 were determined by MTS assay. The mRNA expression levels of GRP78 (*left*), SPARC (*middle*), and the relative expression level (GRP78:SPARC) (*right*) in (C) CRC cell lines (HT29, CCL227, HCT116), (D) MIP101 and (E) RKO 5-FU- and CPT-11-resistant cell lines were determined by q-RT-PCR. Data represent mean ± SEM, N=3.

3.3.4 Low Expression of GPR78 to SPARC ratio Correlates with Improved Survival in Individual with CRC

Using a tissue microarray (TMA) containing CRC tissues of 143 individuals with CRC, the levels of expression of GRP78 and SPARC in CRC tissues were analyzed to determine if there is any correlation between the expression of these individual proteins and disease-free survival in individuals with CRC (Fig. 3.7.A). There was no significant statistical association between the expression level of either GRP78 or SPARC with disease-free survival (data not shown). However, when the relative expression of GRP78 to SPARC was examined, low expression of GRP78/SPARC was associated with a significantly better prognosis: the median disease-free survival of 5.50 months (95% CI: 3.59, 7.45) in comparison to those whose CRC had high GRP78/SPARC expression, median disease-free survival of 3.71 (95% CI: 2.93, 4.30; p-value= 0.01) (Fig. 3.7.B) This result suggests that the low expression of GRP78 to SPARC ratio is associated with improved survival in CRC patients, and may potentially be a prognostic marker for CRC.

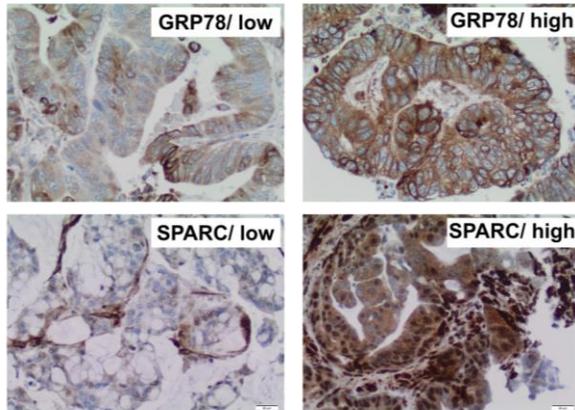
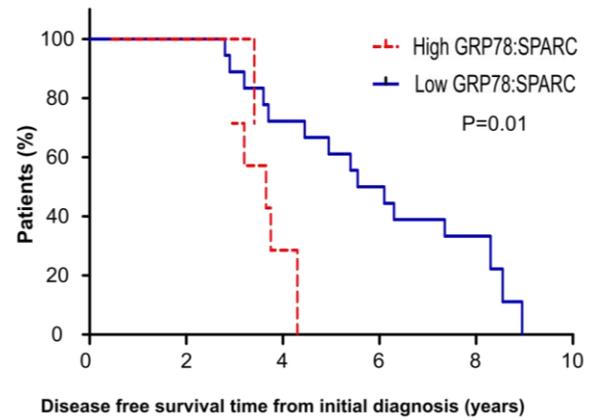
A**B**

Figure 3.7. CRC patients with a low ratio of GRP78:SPARC protein expression have improved prognosis. (A) Representative images of GRP78 and SPARC expression in CRC scored as high expression and low expression; x20, magnification. (B) Kaplan-Meier survival curves of patients with CRC. Individuals with tumor expression low GRP78:SPARC ratio have significantly higher median disease-free survival.

3.3.5 SPARC Sensitizes CRC to PERK/eIF2 α and IRE1 α / XBP-1 ER Stress Signaling

Since GRP78 is a known modulator of ER stress signaling, we next examined the activation of ER stress signaling in the presence of SPARC to understand the mechanism behind the effects of the interaction between GRP78 and SPARC following ER-stress, including chemotherapy. We found that CPT-11 is able to induce ER stress (as shown by an upregulation of GRP78 expression), and activate both PERK/eIF2 α , as demonstrated by the phosphorylation of PERK (Ser713), eukaryotic initiation factor 2-alpha (eIF2 α , Ser51) and the induction of activating transcription factor 4 (ATF4), the downstream transcription factor of phospho-eIF2 α (Fig. 3.8.A). It also activated IRE1 α /XBP-1 ER stress signaling, as shown by the phosphorylation of IRE1 α (S724) and the alternative splicing of XBP-1 in HCT116 cells (Fig. 3.8.B). In line with these observations, the knockdown of SPARC with short interfering RNA attenuated the activation of both PERK/eIF2 α and IRE1 α /XBP-1 signaling (Fig. 3.9).

To further confirm SPARC's ability to influence ER stress signaling, MIP/Zeo and SPARC-overexpressing MIP/SP cells were used to examine these effects of ER-stress signaling following exposure to TM and CPT-11. We observed that in MIP/SP cells, GRP78, phospho-PERK, phospho-eIF2 α and ATF4, were detected at earlier time points than in MIP/Zeo following exposure to TM and CPT-11 (Fig. 3.10.A and B). Importantly, C/EBP-homologous protein (CHOP), a transcription factor that mediates ER-initiated apoptotic cell death, was also induced earlier in MIP/SP treated with TM and CPT-11. Similarly, phospho-IRE1 α were also more prominently expressed in MIP/SP following exposure to tunicamycin and CPT-11 (Fig. 3.11). Moreover, we also examined the apoptotic proteins and found that the expression of the anti-apoptotic protein Bcl-2 is decreased while the expression of the pro-apoptotic Bax increased in MIP/SP, indicating a stronger induction of apoptosis in the SPARC-overexpressing cells under

the drug treatment (Fig. 3.12). Notably, SPARC expression seems to also promote autophagy induction as shown by the increased conversion of microtubule-associated protein light chain 3 (LC3) (LC3-I to LC3-II), although the level of Beclin-1 remains unchanged. Importantly, similar observations of earlier ER stress signaling and apoptosis induction were also observed in MIP/SP exposed to 5-FU (Fig. 3.13). Overall, these data indicate that SPARC may restrict the capacity of CRC cells to sustain ER homeostasis, rendering CRC cells more sensitive to stress stimulus, resulting in earlier activation of ER stress signaling and the downstream apoptotic cascade.

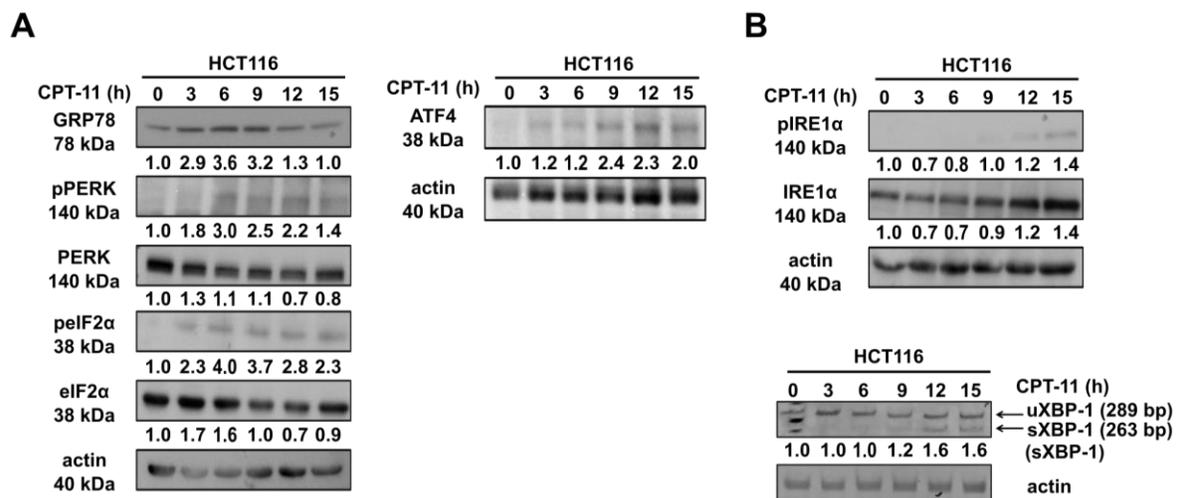


Figure 3.8. SPARC promotes early activation of PERK-eIF2α and IRE1α-sXBP ER stress signaling in endogenous HCT116 cells. HCT116 treated with CPT-11 (50 μM) for various time intervals were analyzed for the presence of the activation of (A) PERK/eIF2α and (B) IRE1α/XBP-1. The spliced form of XBP-1 was detected by RT-PCR analysis. Experiments were repeated (N=3) and the representative images are presented.

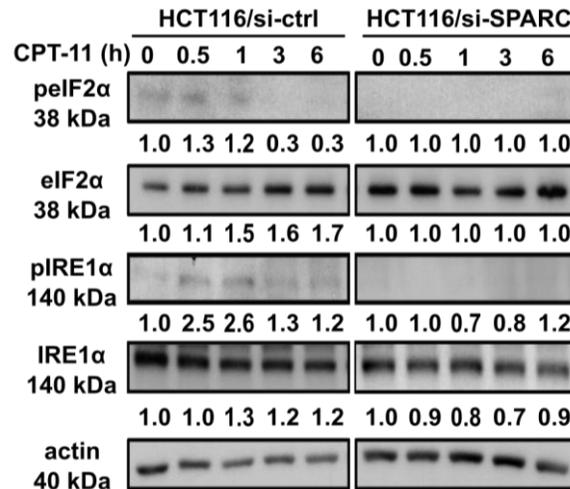


Figure 3.9. Knockdown of SPARC in HCT116 cells suppresses the activation of ER stress signaling induced by CPT-11 treatment. Western blot analysis of ER stress signaling in HCT116 cells following SPARC siRNA knockdown and exposure to CPT-11 (50 μM). Independent experiments were repeated (N=3) and the representative images are presented. Experiments were repeated (N=3) and the representative images are presented.

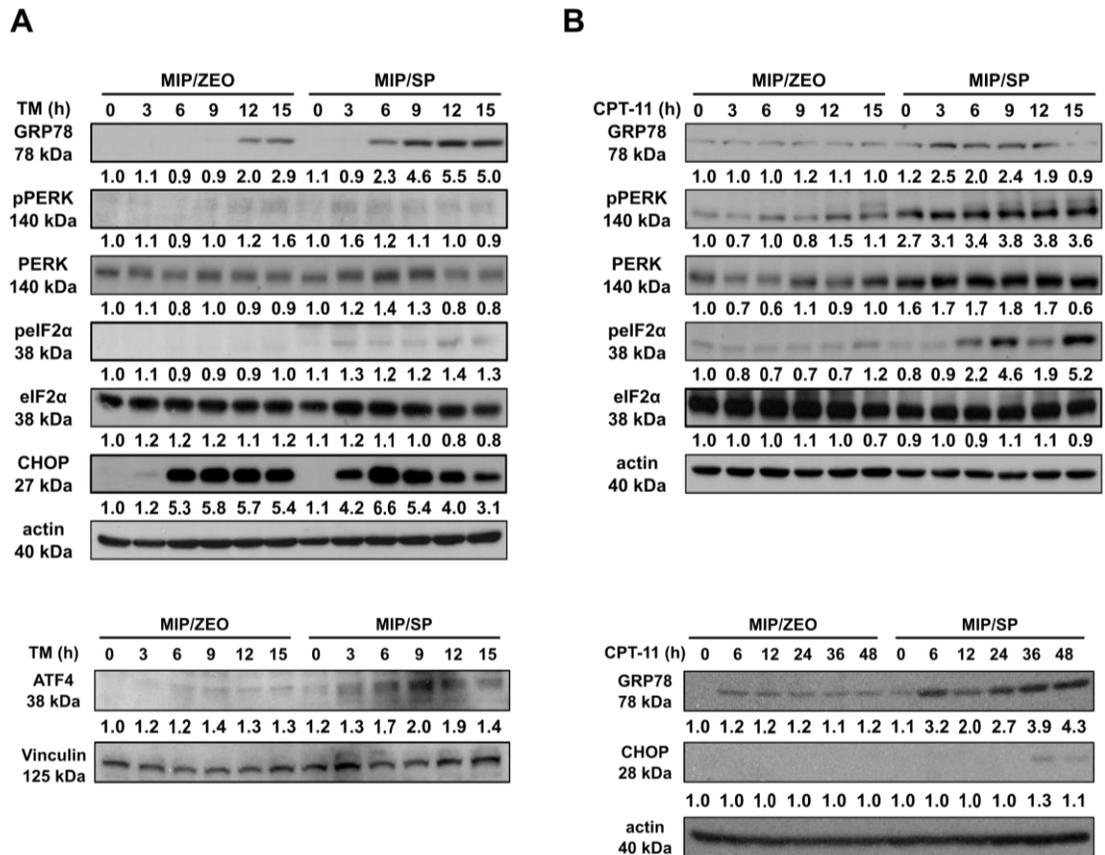


Figure 3.10. SPARC overexpression in MIP101 cells promotes early activation of PERK/eIF2α ER stress signaling. Activation of PERK/eIF2α signaling in MIP/Zeo and MIP/SP (SPARC-overexpressing) following treatment with (A) TM (1 μg/ml) or (B) CPT-11 (50 μM) were examined by Western blot analysis. Experiments were repeated at least two times and the representative images are presented.

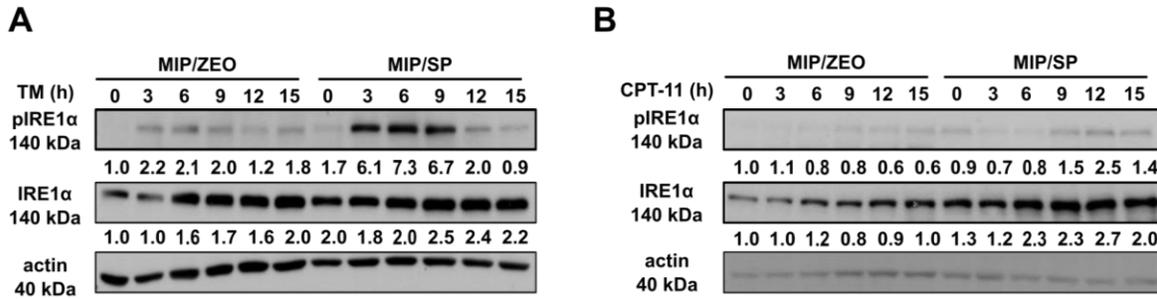


Figure 3.11. SPARC overexpression in MIP101 cells promotes early activation of IRE1α/XBP-1 ER stress signaling. Activation of IRE1α/XBP-1 signaling in MIP/Zeo and MIP/SP following treatment with (A) TM (1 μg/ml) or (B) CPT-11 (50 μM) were examined by Western blot analysis. Experiments were repeated (N=3) and the representative images are presented.

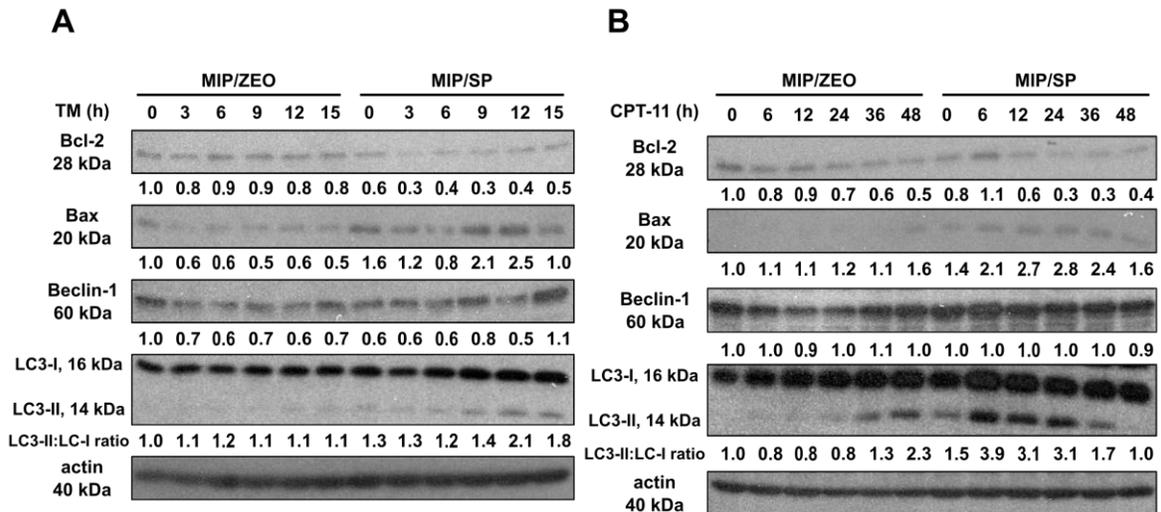


Figure 3.12. SPARC overexpression in MIP101 cells promotes apoptosis and autophagy activation. Apoptosis and autophagy markers were examined in MIP/Zeo and MIP/SP under (D) TM (1μg/ml) or (E) CPT-11 (50μM) treatment. Experiments were repeated at least two times and the representative images are presented.

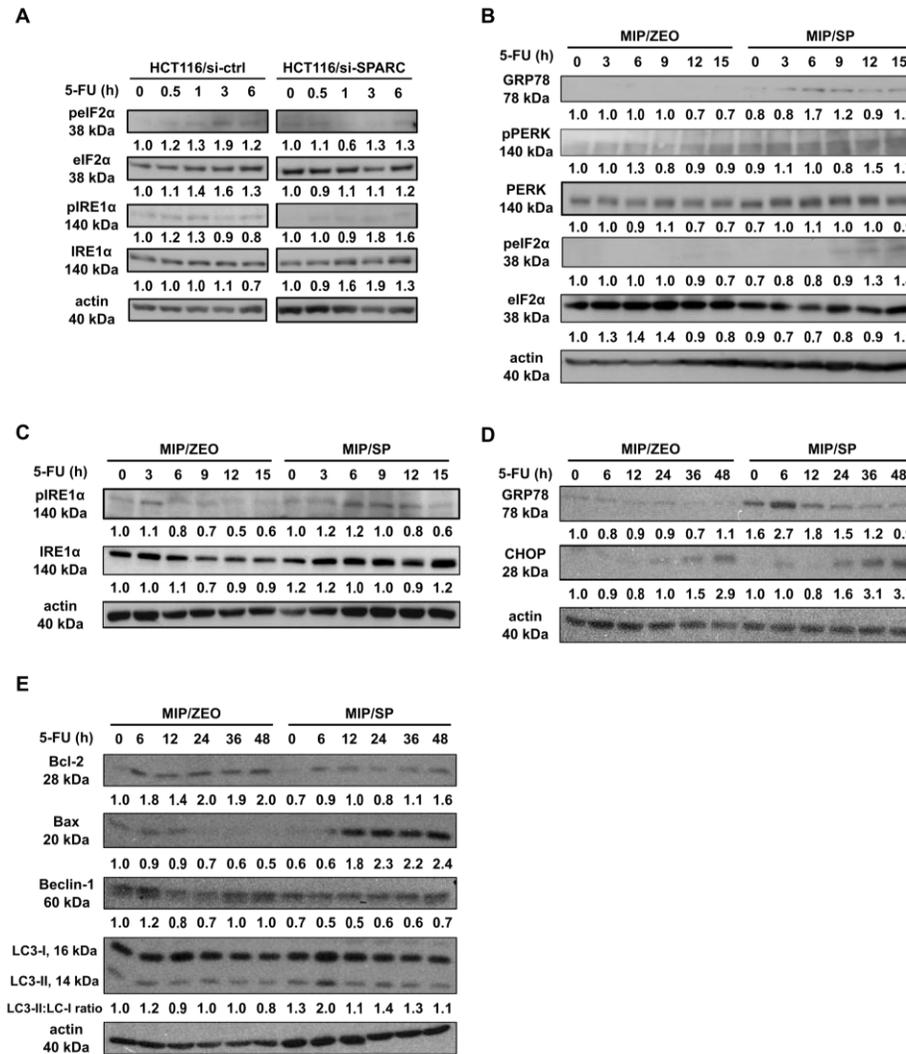


Figure 3.13. 5-FU-induced ER stress and apoptosis in HCT116 and MIP/SP. (A) Western blot analysis showing activation of ER stress signaling in HCT116 cells following SPARC siRNA knockdown and subsequent exposure to 5-FU (25 μ M). Activation of (B) PERK/eIF2 α and (C) IRE1 α /XBP-1 signaling in MIP/Zeo and MIP/SP treated with 5-FU (25 μ M) were examined by Western blot analysis. (D) CHOP induction and (E) expression of apoptosis and autophagy markers were examined in 5-FU-treated MIP/Zeo and MIP/SP cells by Western blot analysis. Experiments were repeated at least two times and the representative images are presented.

3.3.6 SPARC Modulates ER Stress Signaling through Its Interference in the Binding between ER Stress Sensor and GRP78

Under conditions of cellular stress, the dissociation of GRP78 from ER stress sensors leads to the activation of ER stress signaling. We hypothesized that SPARC may sensitize the cells to the activation of ER stress signaling by interfering with the binding between GRP78 and ER stress sensors. To test this hypothesis, we conducted co-IP assays using MIP/Zeo and MIP/SP cells exposed to TM to examine the binding between GRP78 and ER stressors in the presence of SPARC, while under ER stress. We noticed that the level of interaction between GRP78 and PERK was decreased in the MIP/SP cells following exposure to TM (Fig. 3.14.A). Conversely, this interaction only slightly decreased in MIP/ZEO cells following tunicamycin treatment. Similar results were also found in the cells treated with CPT-11 (Fig. 3.14.B). These results suggest that SPARC may weaken the binding between GRP78 and PERK, thereby lowering the threshold of ER stress signaling activation under stress and facilitate ER-stress associated cell death (Fig. 3.15).

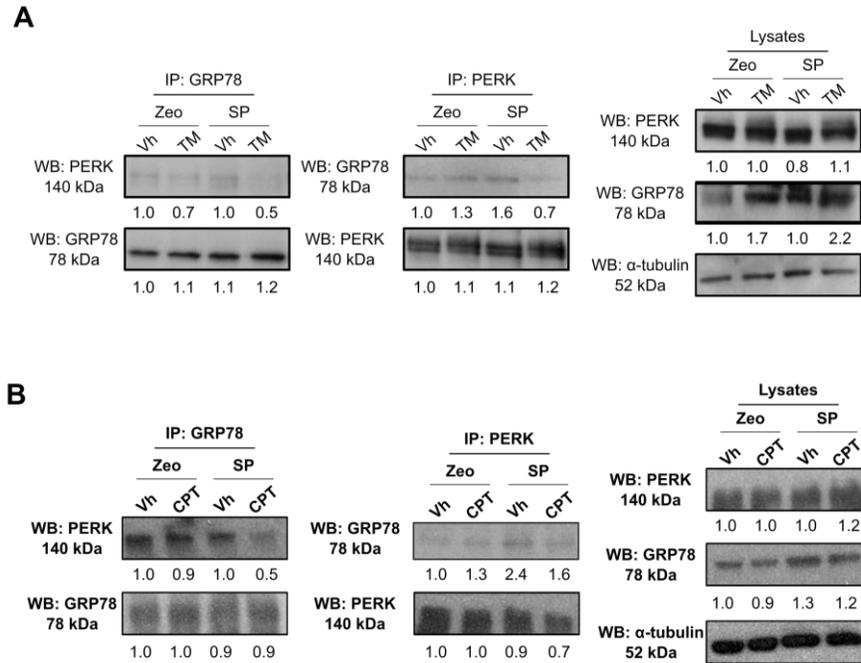


Figure 3.14. SPARC interferes with the binding between GRP78 and PERK under ER stress. MIP/Zeo and MIP/SP were treated with (A) TM (1 μ g/ml, 6 h) or (B) (50 μ M, 12 h) and the cell lysates were immunoprecipitated with anti-GRP78 and anti-PERK antibodies, respectively, followed by Western blot analysis. Experiments were repeated (N=3) and the representative images are presented.

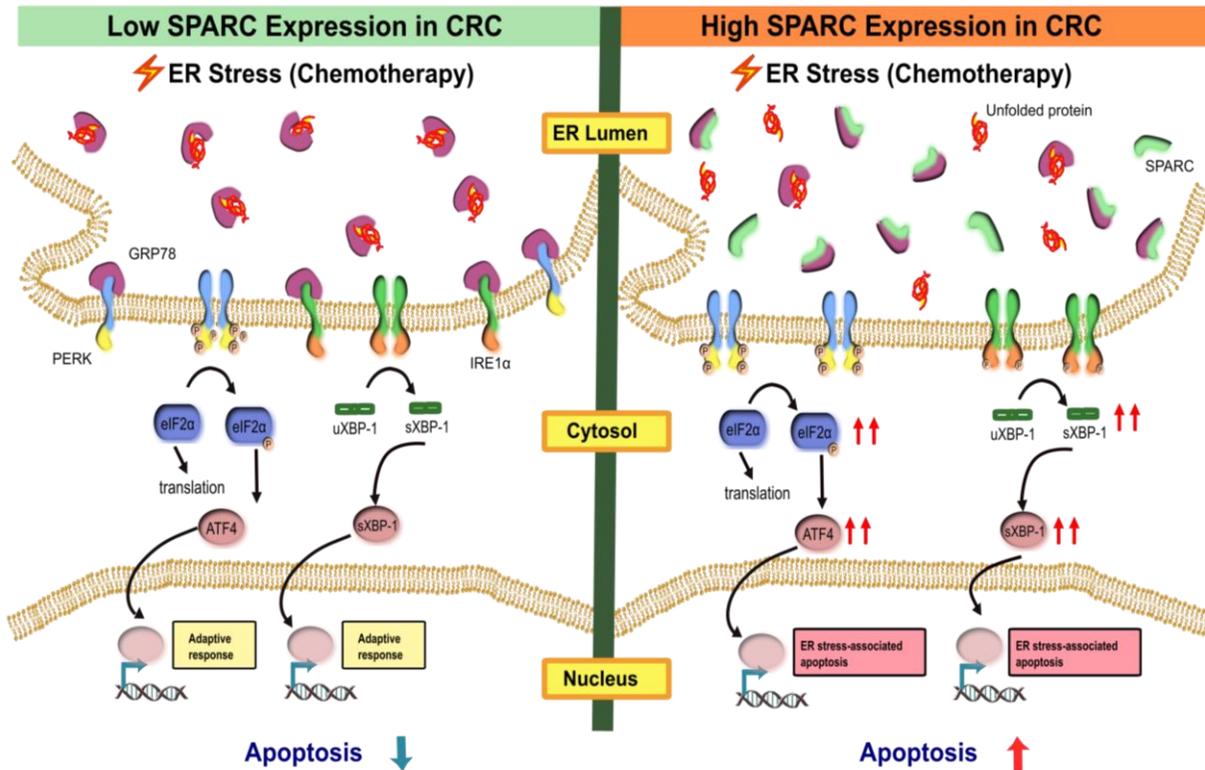


Figure 3.15. SPARC reduces the tolerance to ER stress in CRC cells. SPARC interferes with the binding between GRP78 and ER stress sensors (such as PERK and IRE1 α) via its interaction with GRP78. Under ER stress, the stress stimulus is amplified in high SPARC-expressing cells, as SPARC facilitates the dissociation of GRP78 from the ER stress sensors, thereby lowering the threshold of ER stress signaling and subsequent activation of the apoptotic cascade.

3.4 Discussion

ER stress signaling is often activated in cancer in order to support the rapid growth of cancer cells in a challenging environment that would be inadequate to support the viability of normal cells (173,174). However, this signaling event can be viewed as a double-edged sword: it can support cancer cell survival in an adverse environment while promoting cell death under harsh and sustained stress conditions. Mechanisms determining the switch from adaptive to cell death signaling under ER stress have not yet been clearly defined, and the outcome of the activation of ER stress signaling may depend on the type, intensity and duration of the stimulus in relation to active changes in cellular environment. For example, under mildly stressful cellular conditions PERK-eIF2 α -ATF4 axis may facilitate the expression of genes involved in amino acid transport and glutathione biosynthesis, which facilitates the reestablishment of protein homeostasis in order to support cellular survival (175,176). However, under conditions of severe cellular stress (example: exposure to chemotherapy), PERK signaling can induce the expression of CHOP, which promotes apoptosis by upregulating the expression of proapoptotic BH3-only proteins while suppressing Bcl-2 (176,177). In this study, we show that SPARC serves as a modulator of ER stress: in cancer cells with an abundance of SPARC, this protein lowers the threshold of ER stress signaling activation likely by interfering with the binding between GRP78 and stress sensors. And similar to other proteins that can regulate the magnitude of ER stress signaling, such as BAX, BAK (178) and ASK1-interacting protein 1 (AIP1) (179), SPARC's known association and promotion of apoptosis (134), suggests an important association between apoptosis and ER stress signaling in determining cellular events in cancer.

The involvement of SPARC in ER stress signaling does not appear to be restricted to CRC. For instance, in gliomas, SPARC mRNA undergoes endonucleolytic cleavage by IRE1 α in

response to ER stress (180), and the loss of functional IRE1 α leads to an upregulation of SPARC (181). In the present study, we showed that the expression of SPARC in CRC promotes the activation of PERK and IRE1 α signaling. This suggests that the cleavage of SPARC by activated IRE1 α may serve as a negative feedback mechanism that reduces SPARC level to attenuate the activation of ER stress signaling. We also began an initial assessment of autophagy in relation to ER stress and noticed a greater susceptibility of cells overexpressing SPARC to promote the conversion of LC3-I to LC3-II following ER-stress induced by chemotherapy. Our observation is consistent with previous studies that demonstrated autophagy-mediated apoptosis following radiation therapy-mediated ER stress in neuroblastomas overexpressing SPARC (182). This is not surprising, as the induction of autophagy, similar to ER stress, can either lead to cell death or survival depending on the cellular and/or environmental context (183). Our findings suggest that SPARC may play a key role in coordinating ER stress response and autophagy signaling in cancer cells to facilitate a more favorable response to chemotherapy (184,185).

In contrast to SPARC, GRP78, which is often upregulated on the cell surface in malignancy, can interact with PI3K, CRIPTO, IGF1-R in breast, prostate and hepatoma cells, respectively, to promote oncogenic events (186-190). It is therefore not surprising that the use of an antibody against cell surface GRP78 has been shown to suppress cell survival in chemotherapy-resistant multiple myeloma and glioma cells (186,191). Our demonstration that SPARC, by potentiating ER stress signaling through its interaction with GRP78 to promote cell death following chemotherapy, may be another mechanism that likely contributed to previous reports of dramatic regressions of therapy-resistant CRC tumor xenografts following SPARC-based therapy (133,135). Therefore, a potential strategy in the treatment of CRC may involve the modulation of ER-stress using a combination of SPARC-based and anti-GRP78

based therapies in a more personalized approach. This would tailor the treatment to individuals with CRC that have high GRP78 but low SPARC expression, in a more personalized manner. This sub-population of individuals would most likely benefit from this tailored treatment since our findings also suggest that individuals with CRC that express relatively higher GRP78:SPARC is associated with poorer overall survival.

Our findings also indicate that the expression of GRP78:SPARC may be an effective prognostic biomarker in CRC. Currently, only 30-50% of patients show an objective response to standard FOLFIRI or FOLFOX regimen for metastatic CRC, and disease progression is an expected outcome (192,193). Prognostic and predictive biomarkers that can help identify individuals at higher risk of early relapse and help guide treatment options are greatly needed. SPARC expression has previously been assessed for its utility as a prognostic biomarker, and in line with current observations, high SPARC expression in primary CRC has been shown to be associated with better outcomes, based on longer disease-free survival in stage II and III CRC patients (194). However, the converse has also been shown, as upregulation of SPARC has also been linked to poor outcomes following adjuvant chemotherapy in CRC patients (195). Expression of GRP78 in CRC and its utility as a biomarker has also encountered variable results, as high GRP78 expression has been associated with both good and poor prognosis in CRC patients following chemotherapy (196,197). These studies demonstrating paradoxical findings indicate that single gene markers may not be sufficient to predict chemotherapeutic response, given the complex signaling networks in CRC. Consistent with this notion, we found that while no association was in relation to SPARC or GRP78 expression level and the disease-free survival, the relative expression level of the ratio of GRP78 to SPARC, could predict the clinical outcome for CRC patients.

In summary, this study demonstrates that SPARC modulates ER stress signaling through its interaction with GRP78. Via this mechanism, SPARC further potentiates apoptosis during chemotherapy treatment in CRC by inducing ER stress-associated cell death. Our findings can lead to alternative strategies that can help guide the management of individuals with CRC.

Chapter 4: Heat Shock Protein 47 Promotes Chemoresistance by Modulating AKT Signaling via PHLPP1 in Colorectal Cancer

4.1 Introduction

The primary physiological function of HSP47 is to facilitate the collagen biosynthesis as a collagen-specific chaperone (198) and the dysregulation of HSP47 is frequently associated with collagen-related disorders such as lung and liver fibrosis (148,199). There are limited literature concerning the role of HSP47 in cancer cells. Existing studies indicate that HSP47 may exert functions resulting in both tumor progression and suppression, depending on the cellular contexts (153,158). HSP47 expression has been found to be upregulated in a small number of CRC patients (N=10) and certain ulcerative colitis-associated carcinoma cell lines through mass spectrometry analysis; however, the exact role and the underlying molecular mechanisms by which HSP47 functions in CRC still remains unknown (152,200).

As mentioned in section 3.1, we identified HSP47 as one of the candidate SPARC-binding partners in the mass spectrometry assays. However, as HSP47 is under-characterized in CRC, I began this project by examining the role of HSP47 in CRC, particularly focusing on its effects on the cellular response of CRC cells under chemotherapy. The expression level of HSP47 was firstly investigated in clinical samples as well as online databases. The effects of HSP47 on chemoresistance and the associated molecular mechanisms were subsequently determined *in vivo* and *in vitro*. In summary, HSP47 was found to promote chemoresistance in CRC via Akt signaling, suggesting it as a novel therapeutic target in CRC treatment.

4.2 Materials and Methods

4.2.1 Patients Cohort and Data Analysis

Gene expression (RNA-Seq) data and corresponding clinical data of CRC samples were retrieved from the TCGA data base (<http://genome-cancer.ucsc.edu/>) and curatedCRCData (<http://bioconductor.org/packages/curatedCRCData>). Samples included in the study have pathology-confirmed adenocarcinoma of the colon or rectum, are chemotherapy naive, and have survival information. The survival data were right-censored at 5 years to minimize non-tumor-related causes of death. Follow-up was completed on Sep 10, 2018, in TCGA database and curatedCRCData base.

For the Kaplan-Meier survival curve analysis, we selected the cutoff scores based on receiver operating characteristic (ROC) curve analysis (201). The highest score with maximum sensitivity and specificity on the curve was selected as the cutoff point. The data were dichotomized into high-level and low-level groups, followed by Kaplan-Meier survival analysis (202). To use ROC curve analysis, the clinical outcome and the normalized mRNA expression level index (number of gene transcript was normalized to the total transcript number of the patient) were dichotomized: dead and alive in the follow-up data as clinical outcome, high expression and low expression in the index. The log-rank test was used to compare the difference between two groups. ROC curves and Kaplan-Meier survival curves were analyzed by Survminer package version 0.4.2 under R version 3.4.1 environment.

4.2.2 Plasmids Construction and Stable Cell Line Establishment

For the establishment of RKO/si-ctrl and RKO/si-HSP47 stable cell lines, RKO cells were transfected with pBasi-NC (control) or pBasi-HSP47 plasmids (a gift kindly provided by

Dr. Takehiro Kobayashi (203)) using Lipofectamine 2000 (Invitrogen) followed by puromycin (1.5 $\mu\text{g/ml}$) selection (Thermo Fisher Scientific, MA, USA). The non-effective scrambled shRNA and pGFP-C-sh-HSP47-A plasmids were purchased from OriGene (MD, USA) and used for transient knockdown experiments. For construction of lentiviral vector expressing HSP47, full-length human HSP47 was prepared by PCR amplification of pZeoSV2 (-)-HSP47 plasmid (a gift kindly provided by Dr. Takehiro Kobayashi (203)) and subcloning into the *Bam*HI/*Sa*II sites of pLenti-CMV-GFP-Hygro (Addgene, MA, USA) with GFP gene removal by restriction enzyme digestion to yield pLenti-CMV-HSP47-Hygro. For the establishment of HCT116/GFP and HCT116/HSP47 stable cell lines, HCT116 cells were subjected to selection with hygromycin B (100 $\mu\text{g/ml}$) (Thermo Fisher Scientific) following the virus infection (see below for detail).

4.2.3 Lentiviral Infection

For virus packaging, the pGFP-C-shLenti, pGFP-C-shHSP47-A, pLenti-CMV-GFP-Hygro or pLenti-CMV-HSP47-Hygro were co-transfected at 1:1 ratio with packaging plasmids mix (pCMV-delta R8.74, pRSV-Rev, pCMV-VSV-G, ratio- 4:1:1) into 80% confluent 293T cells (seeded the day before) using TransIT-Lenti transfection reagent (Mirus, MI). The virus-containing media were collected, centrifuged to remove dead cells and filtered using 0.45 μm filters. The lentiviral supernatants were then concentrated by using PEG-it virus precipitation solution (System Biosciences, CA, USA), and stored at -80°C in appropriate aliquots. For virus infection, the concentrated virus was overlaid onto the indicated cell lines in the presence of polybrene (8 $\mu\text{g/ml}$) for 48 h followed by cell viability assay.

4.2.4 Immunoprecipitation

Cells were harvested in cold room and fixed in 0.8% paraformaldehyde/PBS at RT for 20 min and 130mM glycine at RT for 5 min before lysed in the immunoprecipitation lysis buffer [50mM HEPES-KOH, pH7.5, 140mM NaCl, 5mM NaF, 2mM CaCl₂, 10% Glycerol, 0.5% NP-40 and 0.25% Triton-X 100] supplemented with proteinase inhibitor (Sigma-Aldrich, MO, USA). Cell lysates were pre-cleared with normal mouse IgG (Sigma) and protein-L beads overnight at 4°C before incubation with primary antibody. 100µg cell lysates were incubated with 3µg anti-HSP47 antibody (sc-13150, Santa Cruz Technology) or anti-PHLPP1 antibody (sc-390129, Santa Cruz Technology) overnight at 4°C followed by incubation with 60µl protein L beads (sc-2336, Santa Cruz Technology) overnight at 4°C. Beads were pulled down and washed in IP lysis buffer according to the manufacturer's instruction. Proteins were eluted in SDS sample buffer and subjected to Western blot analysis.

4.2.5 Xenograft Study

For tumor xenografts of colorectal cancer cell line: 3 x 10⁶ cells of HCT116/GFP and HCT116/HSP47 were resuspended in 100 µl 1:1 PBS/Matrigel (BD Biosciences, NJ, USA), and injected subcutaneously into the right flank of 8-week-old female nude athymic mice (Simonsen Laboratories) using a 26 ½ gauge needle. Once the tumor was measurable (>25mm³), mice were treated with either PBS or 25 mg/kg 5-FU by intraperitoneal injection. Following 6-days of the treatment-free period, the mice were treated with 5 mg/kg 5-FU every other day. Tumor size was monitored by measuring the length and width with calipers, and volumes were calculated with the formula: $(L \times W^2) \times 0.5$, where L is the length and W is the width of each tumor. Tumors resected from mice were washed twice with sterile PBS and either snap-frozen in liquid nitrogen

or fixed with formaldehyde and paraffin embedded (FFPE) for immunofluorescence analysis. Proteins were extracted from the frozen tumor tissues with TissueLyser (Qiagen, Hilden, Germany). All studies were approved by the Animal Care Committee at the University of British Columbia, Canada (protocol A16-0092).

4.3 Results

4.3.1 Increased HSP47 Expression in the Tumors of CRC Patients

Given the variable expression of HSP47 in different cancers, we began by examining the expression level of HSP47 in tumor tissues from individuals with CRC. To avoid contamination from areas adjacent to the tumors, laser micro-dissection was performed to ensure purity of tumor cells and their paired adjacent normal cells (N=9 pairs) prior to HSP47 mRNA expression analysis by q-RT-PCR. We found that HSP47 is significantly highly expressed in the CRC tumor compared to their adjacent normal tissues (Fig. 4.1.A) ($P < 0.01$).

We also analyzed the HSP47 expression level in the TCGA (The Cancer Genome Atlas) database and found that it was markedly upregulated in the CRC ($P < 0.001$) (Fig. 4.1.B). A similar result was also obtained by analyzing data compiled from 22 independent microarray databases (curatedCRCData, version 2.12.0) ($P < 0.001$) (Fig. 4.1.C) (204). Consistent with the above analysis, the protein expression profiles found in the Human Protein Atlas also revealed that HSP47 is upregulated in the majority of the CRC tissues (High/ Medium staining in 8/12 CRC tissues) (205).

We wondered if the level of HSP47 expression in CRC may be associated with specific clinical parameters, including survival. Individuals with CRC in either the TCGA or the curatedCRCdata cohorts were grouped into low HSP47 expression or high-HSP47 expression

subgroups according to the optimal cutoff value determined by the ROC curve based on HSP47 expression levels. There is no association between HSP47 expression with age, gender, race, tumor stage and tumor site observed in both cohorts (Table 4.1 and 4.2). However, the log-rank test demonstrated that the overall 5-year survival (OS) for patients with low HSP47 expression in tumor tissues was significantly higher than those in high group in both TCGA ($P=0.0021$; HR= 0.50, CI [0.33, 0.76]; Fig. 4.2.A) and curatedCRCData cohorts ($P<0.001$; HR= 2.91, CI [1.92, 4.41]; Fig. 4.2.B). Specifically, individuals with CRC expressing low HSP47 survived 4 months and 23 days longer than those with high HSP47 expression. These results indicate that the expression of HSP47, when upregulated in CRC, may adversely influence the tumor's response to drug treatment.

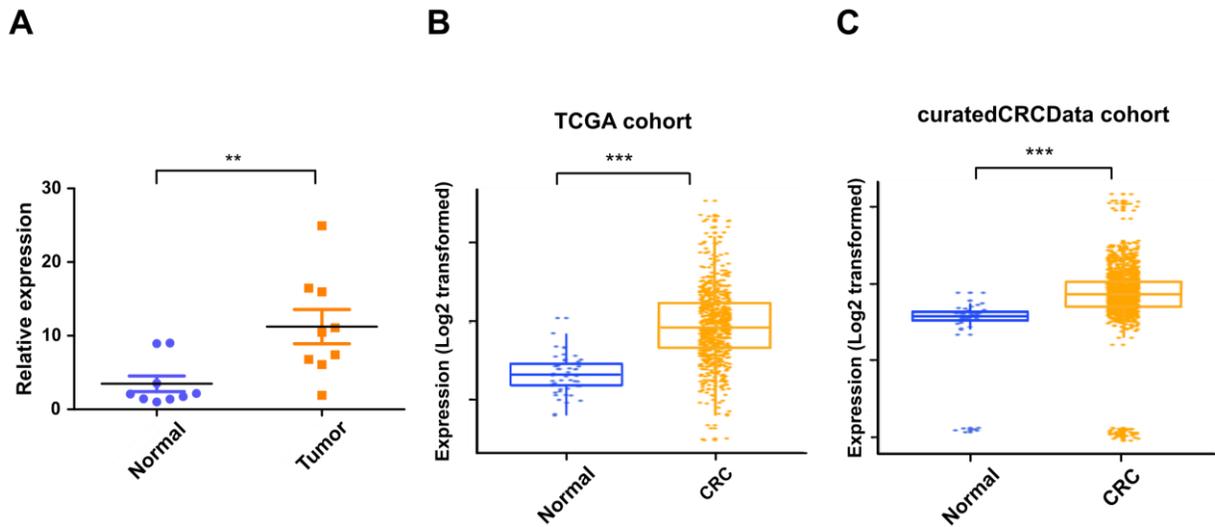


Figure 4.1. Increased expression of HSP47 in colorectal cancer. (A) Real-Time quantitative reverse transcription PCR (q-RT-PCR) analysis of HSP47 mRNA expression level in the paired tumor and adjacent normal tissues of CRC patients (N=9 pairs) collected by laser microdissection ($P < 0.01$); (B) HSP47 mRNA expression level in 644 CRC patients compared with 51 control tissues in TCGA cohort; (C) HSP47 mRNA expression level in 3,296 CRC patients compared with 76 control tissues from 22 microarray databases (curatedCRCData). **, $P < 0.01$; ***, $P < 0.001$.

Table 4.1. Association between HSP47 expression and clinic pathological factors in the TCGA cohort.

Variables	n	High HSP47 (Total N=489)	Low HSP47 (Total N=152)	chi.square	p.value
Gender				0.006	0.940
Male	342	260	82		
Female	299	229	70		
Age				2.594	0.458
<45	38	32	6		
45-59	139	109	30		
60-80	377	280	97		
>80	85	66	19		
Race				11.635	0.020
American native	1	1	0		
Black	68	44	24		
White	311	230	81		
Asian	12	11	1		
Not reported	249	203	46		
Tumor stage				6.283	0.099
I	111	77	34		
II	237	177	60		
III	183	149	34		
IV	90	71	19		
Tumor site				3.612	0.307
Ascending	206	146	60		
Transverse	26	18	8		
Descending	133	99	34		
Rectal	163	129	34		

Table 4.2. Association between HSP47 expression and clinic pathological factors in the curatedCRCData cohort.

Variables	n	High HSP47 (Total N=128)	Low HSP47 (Total N=198)	chi.square	p.value
Gender				0.095	0.758
Male	171	69	102		
Female	155	59	96		
Age				6.852	0.077
<45	22	13	9		
45-59	84	37	47		
60-80	188	64	124		
>80	32	14	18		
Race				0.997	0.802
Black	13	8	5		
White	201	105	96		
Hispanic	2	1	1		
Other	16	10	6		
Tumor_stage				4.060	0.255
I	42	12	30		
II	100	37	63		
III	105	48	57		
IV	79	31	48		
Tumor_site				22.279	2.00E-06
Colon	261	125	136		
Rectal	33	1	32		

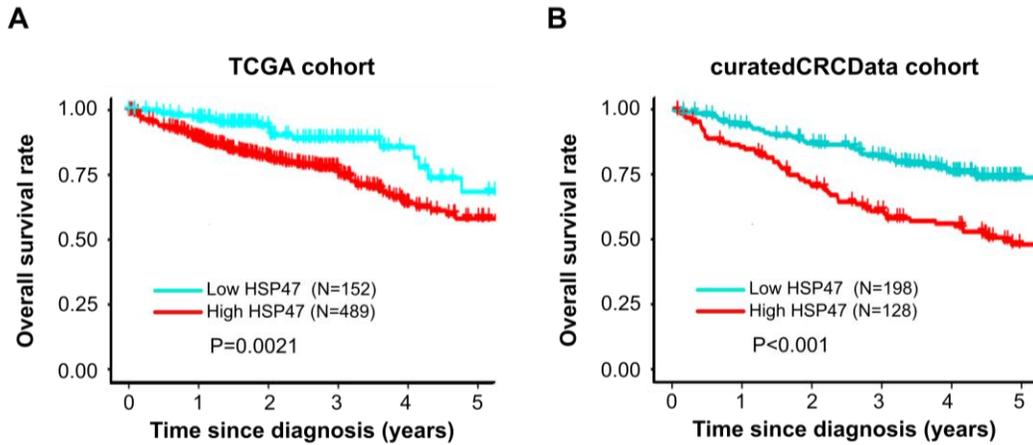


Figure 4.2. CRC patients with high HSP47 expression level have lower overall survival.

Kaplan-Meier (KM) survival analysis of the CRC patients in the (A) TCGA and (B) curatedCRCData cohorts. The patients were divided into high- and low- expression group based on the cutoff value derived from the ROC analysis. **, $P<0.01$; ***, $P<0.001$.

4.3.2 HSP47 Expression Promotes the Resistance of CRC Cells to Chemotherapy

To investigate whether HSP47 influences the response of CRC cells to chemotherapy, we began by examining the viability of CRC cells following the modulation of HSP47 expression level and drug exposure. We selected three p53-wild type CRC cell lines (HCT116, CCL228 and RKO) based on their HSP47 expression level by Western blot analysis (Fig. 4.3). Following the transient transduction with HSP47 overexpressing or knockdown lentiviral vectors (Fig. 4.4), the three CRC cell lines- HCT116, RKO and CCL228- were exposed to various concentrations of 5-FU followed by MTS assay analysis. We found that reduced HSP47 expression level increased the sensitivity of RKO and CCL228 cells to 5-FU treatment. There was a 5.5 ± 1.8 and 7.0 ± 1.2 % reduction in cell viability after 5-FU ($24\ \mu\text{M}$) treatment in the HSP47-knockdown RKO and CCL228 cells, respectively, (Fig. 4.5 A and B). On the other hand, higher HSP47 expression reduced the sensitivity of CRC cells despite incremental concentrations of 5-FU. For example, there was 10.1 ± 3.0 and 13.3 ± 3.6 % increase of cell viability following 5-FU ($24\ \mu\text{M}$) treatment in HSP47-overexpressing RKO and CCL228 cells, respectively (Fig. 4.6.A and B), and a 5.7 ± 1.6 % increase in cell viability after 5-FU ($16\ \mu\text{M}$) exposure in HCT116 cells overexpressing HSP47 (Fig. 4.6.C).

In RKO and HCT116 cell lines with established resistance to 5-FU (RKO/5FU, (Fig. 4.7.A); HCT116/5FU, Fig. 4.7.B;) (135) the basal levels of HSP47 mRNA and protein expression determined by q-RT-PCR and Western blot analysis, respectively, were significantly higher than the sensitive parental cell line.

Combined these data suggest that HSP47 may influence the response of CRC cells to chemotherapy.

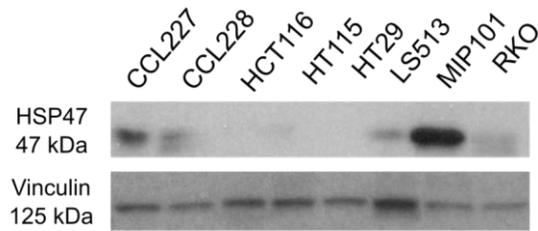


Figure 4.3. HSP47 protein expression in CRC cell lines. Representative image of Western blot analysis of HSP47 protein expression in various human CRC cell lines. Experiments were repeated three times and the representative images are presented.

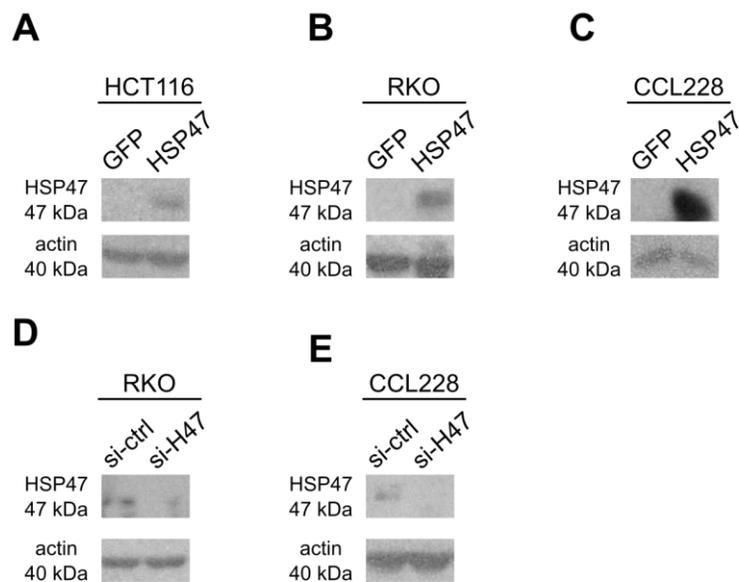


Figure 4.4. Examination of HSP47 expression level in CRC cell lines with HSP47 transient overexpression or knockdown. Western blot analysis of HSP47 expression level in (A) HCT116, (B) RKO cells and (C) CCL228 cell lines after 48, 48 and 72 hrs transient transduction, respectively, of HSP47-overexpressing vectors. Western blot analysis of HSP47 expression level in (D) RKO and (E) CCL228 cells after 48 hrs transient knockdown of HSP47.

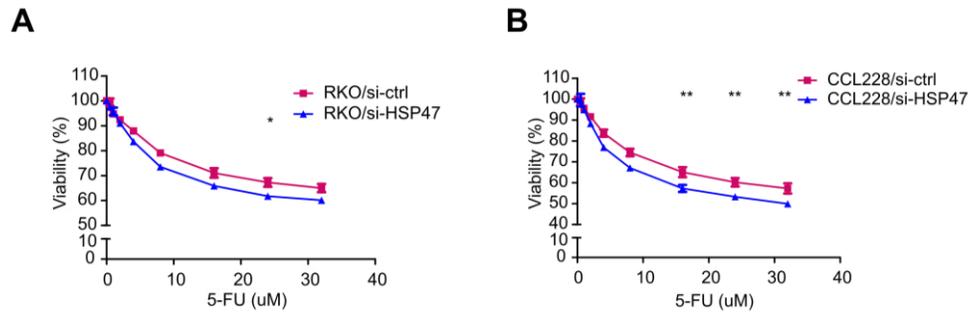


Figure 4.5. Transient knockdown of HSP47 sensitized RKO and CCL228 CRC cell lines toward chemotherapy. MTS cell viability assays of (A) RKO and (B) CCL228 cells exposed to various concentration of 5-FU following HSP47 transient knockdown. Data represent mean \pm SEM, N=3. *, $P<0.05$; **, $P<0.01$.

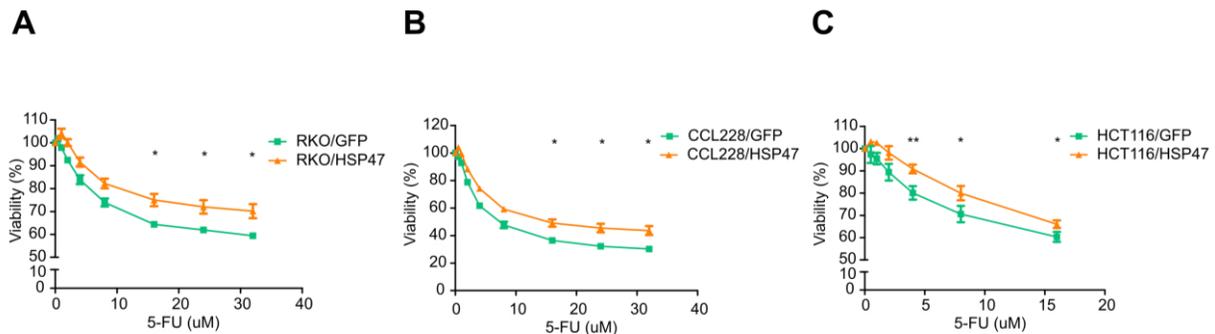


Figure 4.6. Transient expression of HSP47 increases the resistance of RKO, CCL228 and HCT116 CRC cell lines under chemotherapy. (A) RKO, (B) CCL228, and (C) HCT116 CRC cell lines were transiently transduced with HSP47-expressing vector followed by exposure to 5-FU. Cell viability was determined by MTS assay. Data represent mean \pm SEM, N=3. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

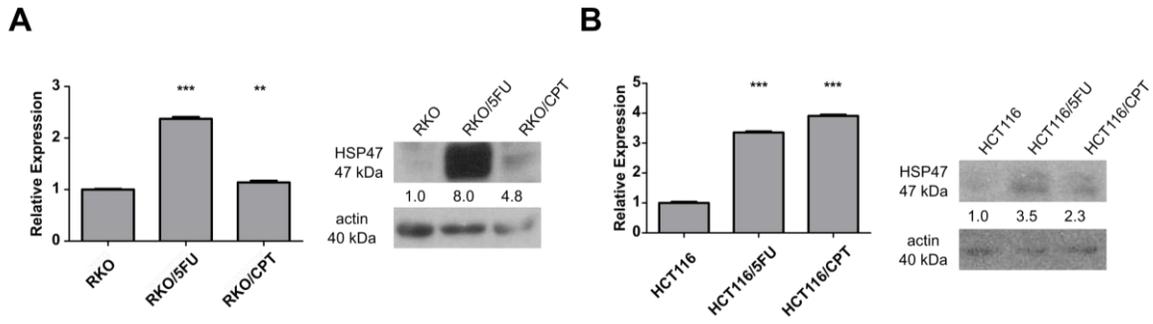


Figure 4.7. Increased HSP47 mRNA and protein expression in CRC resistant cell lines.

HSP47 mRNA and protein expression in (A) RKO and (B) HCT116 resistant cell lines were determined by q-RT-PCR and Western blot analysis, respectively. Data represent mean \pm SEM, N=3. **, $P < 0.01$; ***, $P < 0.001$.

4.3.3 HSP47 Inhibits the Apoptosis Induction of CRC Cells under Chemotherapy

As apoptosis influences, in part, clinical response to drug treatment, we further explored the effects of HSP47 on the apoptotic pathway by conducting dUTP-digoxigenin nick end labeling (TUNEL) assay on HSP47-overexpressing HCT116/HSP47 cells and its vector control (HCT116/GFP). We observed that the percentage of TUNEL-positive cells were significantly lower in HCT116/HSP47 cells (Vehicle: 1.0 ± 1.5 %; 5-FU: 5.3 ± 3.9 %) compared to that in the control cell line (Vehicle: 5.3 ± 5.0 %; 5-FU: 52.4 ± 9.9 %) when the cells were exposed to 5-FU. This indicated that there was a reduction in the number of cells undergoing apoptosis when cells overexpressed HSP47 (Fig. 4.8.A and B). In line with this observation, there was a similar reduction in the expression of cleaved caspase-3 and -7 in HCT116/HSP47 exposed to 5-FU (Fig. 4.9.A). Consistent results were also observed in the HSP47 stable-knockdown RKO cells (RKO/si-HSP47): cleaved caspase-3 and -7 were detected at earlier time points following exposure to 5-FU (Fig. 4.9.B). These results suggest that the expression of HSP47 may inhibit the apoptotic pathway, thereby contributing to the drug resistance in CRC cells in response to chemotherapy.

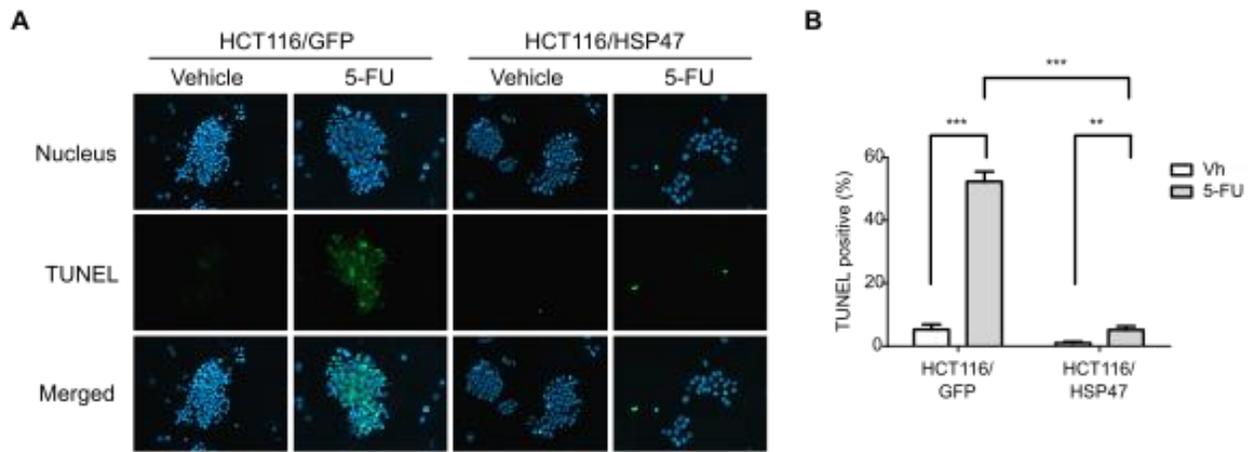


Figure 4.8. HSP47 expression reduces TUNEL positive cells in HCT116 exposed to 5-FU.

(A) HCT116/HSP47 cells were visualized by fluorescence microscopy (200X) at 48 h after 50 μ M 5-FU treatment. TUNEL-positive nuclei are shown in green, total nuclei stained with Hoechst 33258 in blue. (B) Quantitative analysis (percentage of TUNEL positive cells versus total) was performed with randomly selected fields (N=10). **, $P < 0.01$; ***, $P < 0.001$.

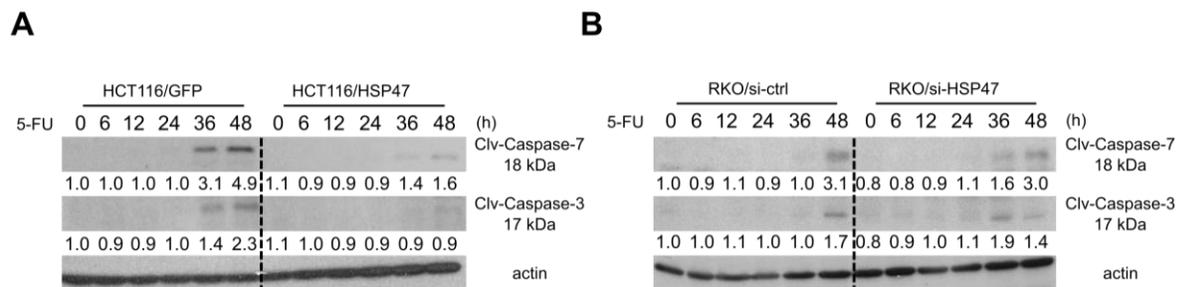


Figure 4.9. HSP47 expression suppresses the cleavage of caspase-3 and -7 in HSP47-

overexpressing and knockdown cell lines. Representative Western blot images of caspase-3 and -7 cleavages of (A) HCT116/HSP47, (B) RKO/si-HSP47 and their corresponding control cell lines after treatment with 50 μ M 5-FU. Experiments were repeated at least two times and the representative images are presented.

4.3.4 HSP47 Promotes the Phosphorylation of AKT at Ser 473 in CRC Cells under Chemotherapy

The dysregulation of the Akt/PKB pathway has been demonstrated to substantially contribute to therapy-refractory disease in a variety of cancers, including CRC (206-209). In addition to the anti-apoptotic effects exerted by HSP47, we were interested in examining the effect of HSP47 on Akt signaling. Western blot analysis following the treatment of HCT116/HSP47 (Fig. 4.10.A) and RKO/si-HSP47 (Fig. 4.10.B) with 5-FU demonstrated increased phosphorylation of Akt at Ser 473 in cells with higher HSP47 expression level (Fig. 4.11.A and B). However, when we examined the activation of Akt kinase mTORC2 (41), changes in the expression level of p-mTOR-S2481 (mTORC2 activation marker (210)), did not reflect the phosphorylation level of Akt at S473. Instead, we noticed higher expression of Akt-S473 phosphatase PHLPP1(43) that persisted for a longer period of time in the cell lines with reduced HSP47 expression (Fig. 4.12.A and B). This suggests that the changes in the phosphorylation level of Akt-S473 in cells with variable expression of HSP47 likely involves PHLPP1.

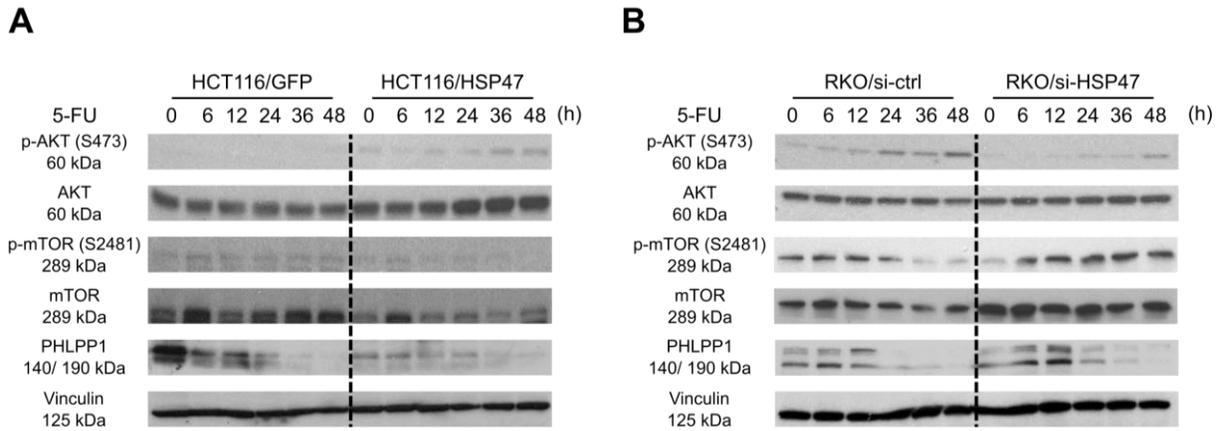


Figure 4.10. HSP47 promotes Akt activation and reduces PHLPP1 expression in CRC cells exposed to 5-FU. Representative immunoblotting images of (A) HCT116/HSP47, (B) RKO/si-HSP47 and their corresponding control cell lines treated with 50µM 5-FU for the indicated time periods.

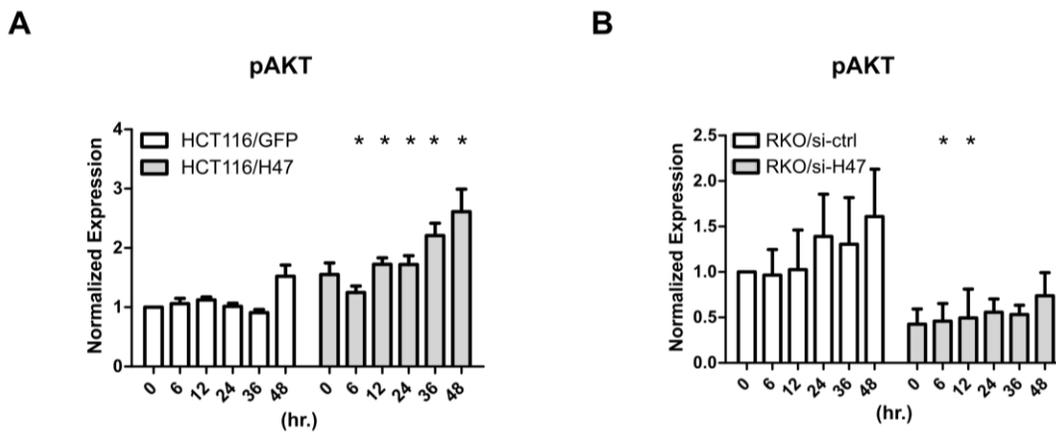


Figure 4.11. Quantification of immunoblotting images of p-Akt (S473) in HCT116/HSP47 and RKO/si-HSP47 exposed to 5-FU. The immunoblotting images of phosphor-Akt (S473) in (A) HCT116/HSP47, (B) RKO/si-HSP47 and their respective control cell lines were quantified with Image J software (N=3). Data represent mean ± SEM. *, $P < 0.05$.

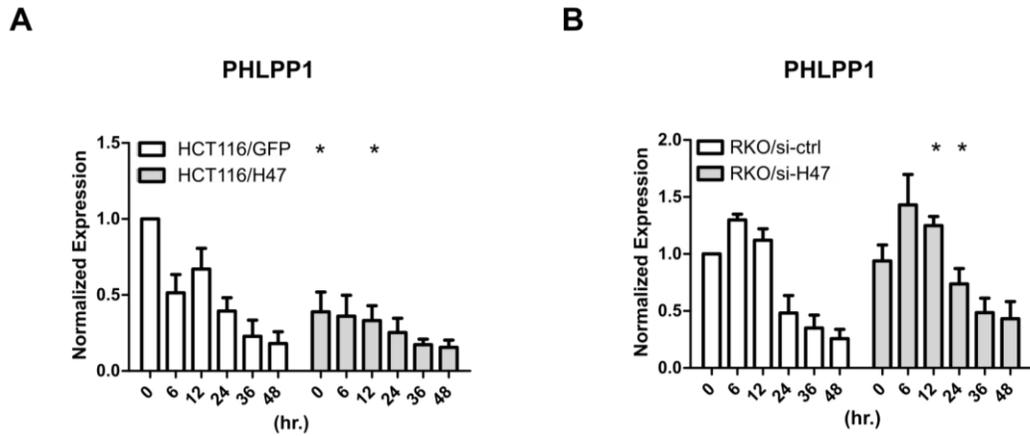


Figure 4.12. Quantification of immunoblotting images of PHLPP1 in HCT116/HSP47 and RKO/si-HSP47 exposed to 5-FU. The immunoblotting images of PHLPP1 in (A) HCT116/HSP47, (B) RKO/si-HSP47 and their respective control cell lines were quantified with Image J software (N=3). Data represent mean \pm SEM. *, $P < 0.05$.

4.3.5 HSP47 Interacts with PHLPP1 and Decreases Its Protein Stability

To understand how HSP47 influences PHLPP1 expression in CRC, we also examined the mRNA level of PHLPP1 in the cells by q-RT-PCR analysis. We observed the fluctuations of PHLPP1 mRNA level in both cell lines during the treatment period; however, the alteration of the PHLPP1 mRNA level did not reflect its protein expression pattern during the treatment, suggesting that HSP47 may modulate PHLPP1 expression in a posttranscriptional manner (Fig. 4.13.A and B). To examine this hypothesis, we conducted cycloheximide chase assays to determine whether HSP47 would affect the protein stability of PHLPP1. RKO/si-HSP47 cells were exposed to cycloheximide to suppress further protein (PHLPP1) synthesis, followed by assaying PHLPP1 proteins by immunoblotting. As shown in Fig. 4.14, 66.0 ± 8.5 % of PHLPP1 remained in the RKO/si-HSP47 cells while only 26.7 ± 13.3 % of PHLPP1 were observed in RKO/si-ctrl cells by 4-h cycloheximide exposure, according to the result of densitometry analysis (Fig. 4.14).

Given that HSP47 may be influencing the stability of PHLPP1, we next examined if these two proteins may be interacting. Interestingly, immunofluorescence analysis showed that HSP47 and PHLPP1 colocalized in the RKO parental cell line (Fig. 4.15.A). The interaction between the endogenous HSP47 and PHLPP1 proteins was then validated by co-immunoprecipitation assay (Fig. 4.15.B). These results demonstrate that HSP47 interacts with PHLPP1 to reduce its protein stability, thereby allowing Akt to remain phosphorylated at S473. This prolongs the activation of Akt signaling and promotes cell survival and drug resistance.

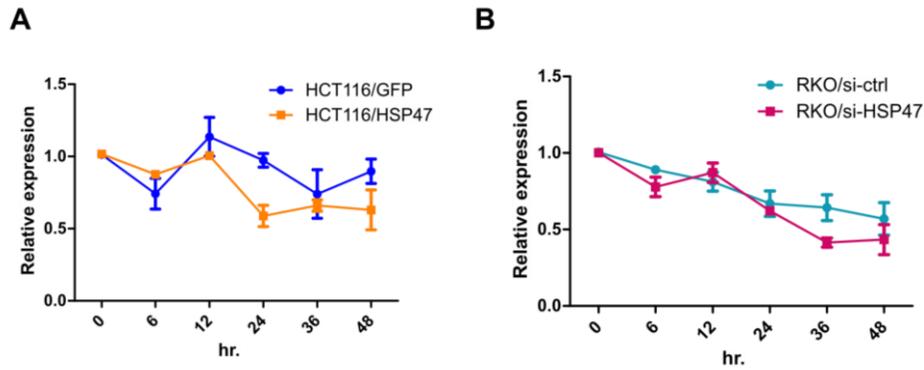


Figure 4.13. PHLPP1 mRNA expression in HCT116/HSP47 and RKO/si-HSP47 exposed to 5-FU. q-RT-PCR analysis of PHLPP1 mRNA expression level in (A) HCT116/HSP47, (B) RKO/si-HSP47 and their corresponding control cell lines treated with 50 μ M 5-FU (N=3). Data represent mean \pm SEM. *, $P < 0.05$.

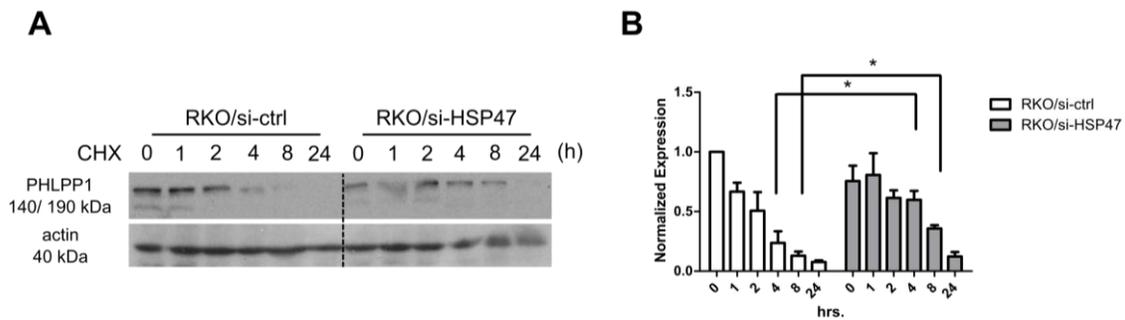


Figure 4.14. HSP47 knockdown increases PHLPP1 protein stability in the cycloheximide chase assay. (A) Representative Western blot images of RKO/si-HSP47 and its control cell line treated with 100 μ g/ml cycloheximide (CHX). Cells were collected at indicated time points followed by Western blot analysis. The immunoblotting images were quantified with Image J software (N=3). (B) PHLPP1 protein expression levels at different time points in the CHX chase assay were quantified by Image J software (N=3). Data represent mean \pm SEM. *, $P < 0.05$.

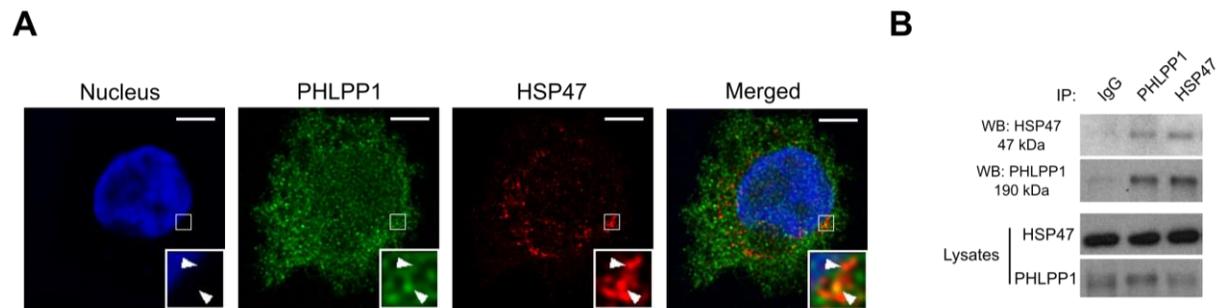


Figure 4.15. HSP47 colocalizes and interacts with PHLPP1 in RKO CRC cell line. (A) RKO cells were double stained with the mouse monoclonal antibody against PHLPP1 (green) and rabbit monoclonal antibody against HSP47 (red) followed by confocal immunofluorescence analysis. Colocalization between PHLPP1 and HSP47 indicated by arrows. (B) Co-immunoprecipitation of HSP47 with PHLPP1 in parental RKO cells. Experiments were repeated (N=3) and the representative images are presented. Scale bar= 5 μ m.

4.3.6 HSP47 Promotes Tumor Survival under Chemotherapy via AKT Activation in CRC Xenograft Model

To investigate whether HSP47 influences tumor survival and therapy resistance *in vivo*, the stable HSP47-overexpressing HCT116/HSP47 and the control cells were subcutaneously injected into the nude mice. The mice were initially treated with 5-FU at 25 mg/kg followed 6 days later with the treatment of 5-FU at 5 mg/kg every second day. By day 16 of implantation (8 days following initiation of treatment), the average tumor volume of HCT116/HSP47-bearing mice treated with 5-FU was significantly larger than the control cell lines exposed to 5-FU (control: 768.95 ± 39.08 ; HSP47: 1850.79 ± 77.05 (mm³), N=5 per group, P<0.001) (Fig. 4.16.A). Importantly, Western blot analysis of the proteins extracted from the tumor tissues showed increased p-AKT (S473) expression in HSP47-overexpressing tumors, while PHLPP1 expression was decreased in the same tumors (Fig. 4.16.B). In addition, immunofluorescence analysis of FFPE xenograft tissues also demonstrated colocalization between HSP47 and PHLPP1 (Fig. 4.16.C). These results support our *in vitro* findings that HSP47 promotes the survival of CRC tumors and drug resistance following exposure to chemotherapy *in vivo*, by enhancing AKT signaling likely via PHLPP1 (Fig. 4.17).

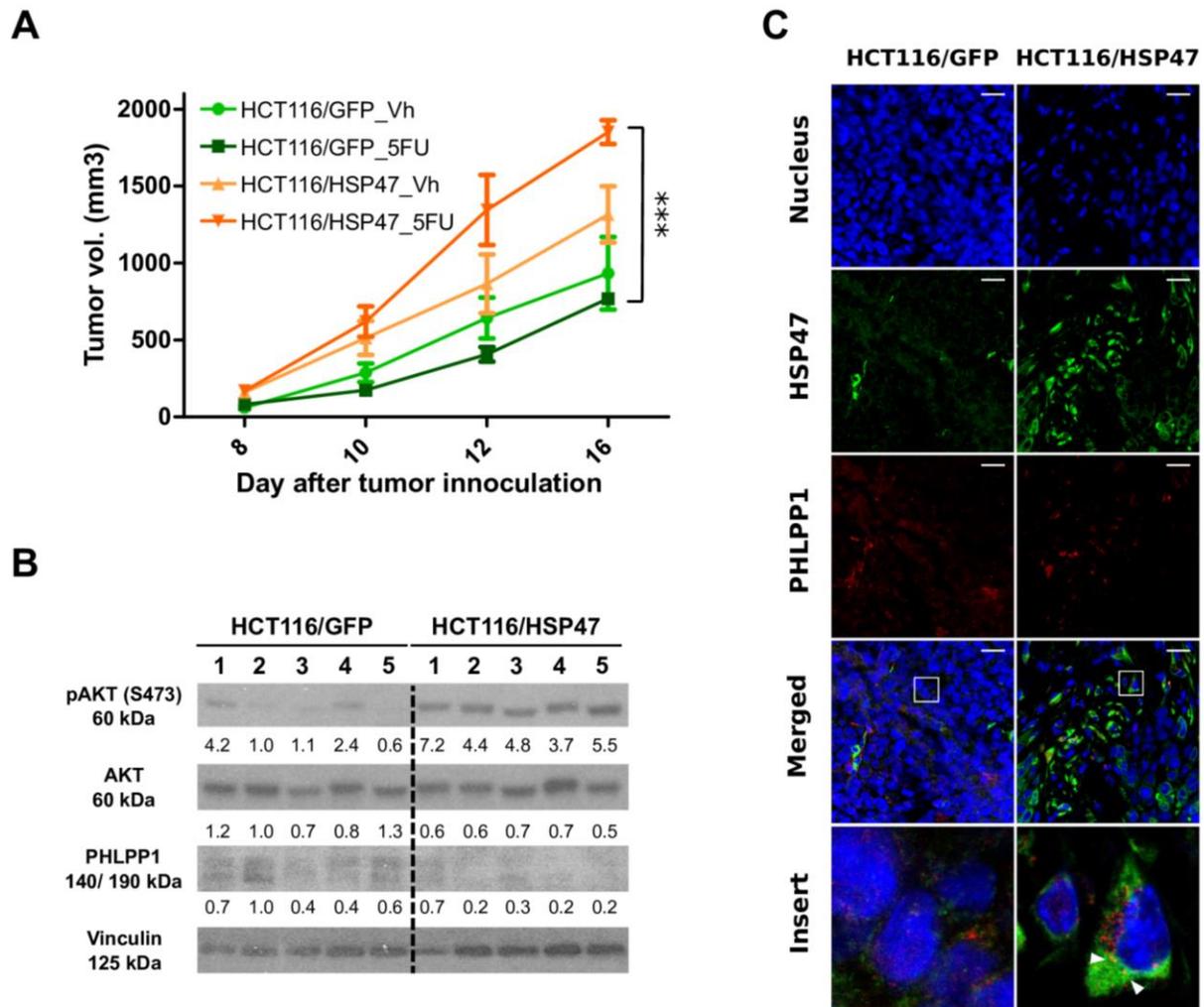


Figure 4.16. HSP47-overexpressing CRC tumors fail to respond to chemotherapy. (A) *In vivo* growth of subcutaneous mouse xenografted tumors of HCT116/HSP47_{st} and the control cell line treated with either vehicle (Vh) or 5-FU, (N=5 per group); (B) Western blot analysis of proteins extracted from tumor tissues recovered from vehicle-treated mice. (C) FFPE xenograft tissues were double stained with the mouse monoclonal antibody against PHLPP1 (red) and rabbit monoclonal antibody against HSP47 (green) followed by confocal immunofluorescence analysis. Note: arrows indicate the colocalization between PHLPP1 and HSP47. Scale bar= 20 μ m. Data represent mean \pm SEM. ***, $P < 0.001$.

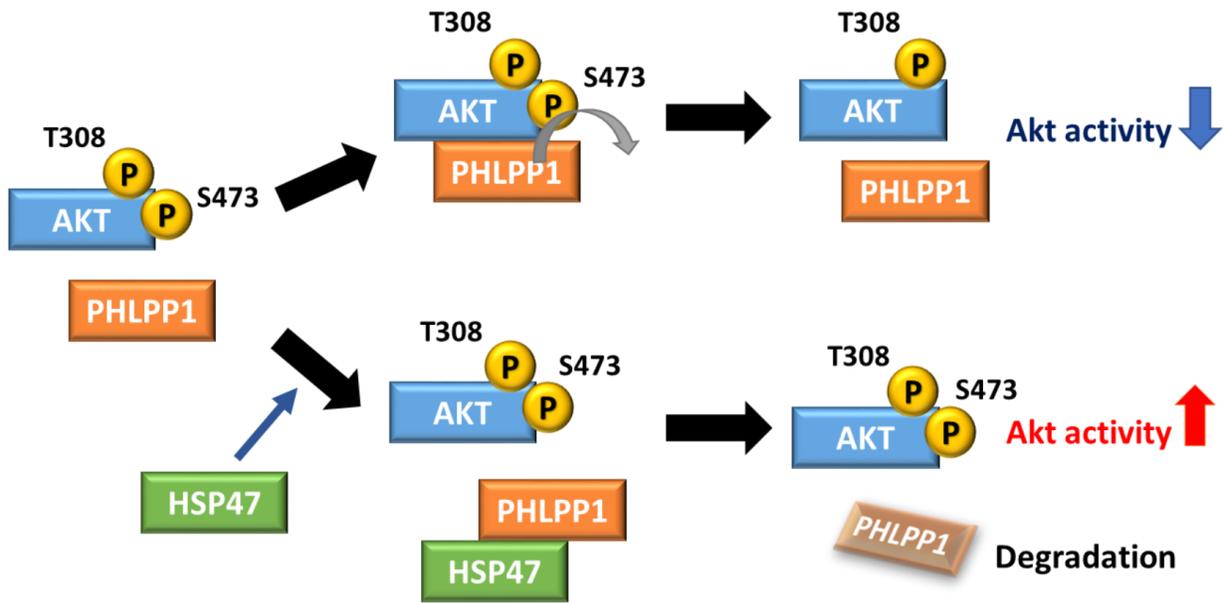


Figure 4.17. HSP47 promotes chemoresistance in CRC through enhancing Akt activation.

Signaling events suggested by this study: HSP47 reduces protein stability of PHLPP1 and promotes Akt activity.

4.4 Discussion

Treatment failure of chemotherapy primarily results from the emergence of drug resistance. Several chemotherapeutic agents, including 5-FU, doxorubicin and paclitaxel, have been shown to modulate PI3K/Akt/mTOR (PAM) signaling, and the activation of PAM pathway is implicated in the resistance to anticancer therapies (211,212). Several studies are ongoing to explore the anti-tumor effects of PAM signaling inhibitors in combination with current regimens (211,213,214). However, the complicated network of interaction with parallel cascades in PAM signaling allows cancer cells to continue to evade PAM inhibition by involving negative feedback mechanisms and compensatory signaling pathways. As examples, both lung and breast cancer cell lines and patient tumors eventually evade mTOR inhibition following exposure to rapamycin derivative, RAD001, by increasing Akt phosphorylation through the upregulation of IGF-1 signaling (215,216). Moreover, increased pAkt (S473) was also found in the tumors of CRC and breast cancer patients following mTOR inhibitor Everolimus treatment (217). These studies suggest that instead of targeting mTOR kinase downstream of PAM signaling, direct inhibition on Akt kinase may be advantageous by avoiding a feedback loop that in turn mediates overaction of Akt. Attempts to develop Akt-specific and isozyme selective inhibitors have also proven difficult, given the high degree of homology in the ATP binding pocket between AKT, protein kinase A (PKA) and protein kinase C (PKC) (218). However, in this study, we show a potential mechanism for targeted Akt inhibition. We show that HSP47 augments PAM signaling in CRC cells in vitro and in vivo, especially in the presence of chemotherapy. This can be reversed by reducing HSP47 expression by shRNA knockdown, resulting in inhibition of Akt and further decline in cell viability in response to chemotherapy. These exciting findings indicate that HSP47 is a potential novel target that can be used to inhibit Akt signaling in CRC.

Moreover, as targeting HSP47 suppresses Akt activity potentially by providing sustained PHLPP1 phosphatase activity, it may also help minimize the development of resistance mechanisms, as seen with mTOR inhibitors

Another benefit of suppression of Akt activity via HSP47 targeting is that, by its direct interaction with PHLPP1, it specifically targets Akt2 and Akt3(44). Although all three isoforms of Akt (Akt1, Akt2 and Akt3) are reported to express in both normal and CRC tissues (219), Akt2 is more abundantly overexpressed in late-stage CRC and metastatic tumors, suggesting that Akt2 plays a critical role in CRC progression (220) and may be more susceptible to HSP47 inhibition. It remains to be explored whether HSP47 also interacts with PHLPP1's isoform PHLPP2, which negatively regulates the activity of Akt1 and 3 (44). Provided that HSP47 is a sole inhibitor of PHLPP1, a potential compensatory response which leads to the hyperactivation of Akt1 may be induced in HSP47-targeted therapy due to the synergistic and overlapping functions of Akt1 and Akt2 in colorectal cancer (221), and this will need to be further examined.

HSP47 is highly expressed and is associated with the abnormal collagen deposition in the myofibroblasts and type II pneumocytes of lungs in individuals with idiopathic pulmonary fibrosis (IPF) tissues (222,223). Although the role of HSP47 is unclear in IPF, inhibition of HSP47 (using a lipid nanoparticle encapsulating a siRNA against HSP47, ND-L02-s0201) has been shown to improve pulmonary fibrosis in phase I and II clinical trials (224). This suggests that specific inhibition of HSP47 is a feasible target in CRC.

Recently HSP47 was identified as an ER stress modulator that binds to ER stress sensor IRE1 alpha and adjusts the activating threshold of ER stress signaling. Downregulation of HSP47 sensitizes both cells and animals to experimental ER stress (150). As ER stress has also been demonstrated to negatively regulate PAM signaling (225), targeting HSP47 may negatively

regulate PAM signaling not only through its interaction with PHLPP1, but also by its effects on ER stress induction. However, it is crucial to investigate how the inhibition of HSP47 affects the dynamic of its dual roles in both PAM and ER stress signaling in the setting of CRC. This is underscored by the fact that the activation of ER stress signaling can lead to either cell survival or ER stress-associated cell death induction, depending on the type and intensity of ER stress (117). Nevertheless, a potential strategy to overcome the pro-survival effects of HSP47 inhibition is to combine it with drugs inducing ER stress, such as bortezomib, to dictate the stress signaling to apoptosis and promote treatment efficacy. The type and dose of drugs to be combined with HSP47-targeted therapy will need to be empirically tested in preclinical studies first. Additionally, it has been demonstrated that Akt2 has been implicated in the establishment of metastasis in CRC. It remains to be explored if targeting HSP47 might also reduce the metastatic ability of CRC as PHLPP1 is an Akt2, 3-specific phosphatase (220).

In summary, we identified HSP47 as a novel protein that promotes cancer survival by modulating Akt signaling via PHLPP1 in colorectal cancer. HSP47 can serve as a potential therapeutic target in CRC to promote the efficacy of chemotherapy.

Chapter 5: Conclusion

5.1 Summary of Study and Findings

5.1.1 Chapter 3

In Chapter 3, we demonstrated that SPARC sensitizes CRC cells to chemotherapy by modulating ER stress signaling to promote ER stress-associated cell death. We identified a novel interaction between SPARC and GRP78 and found that this interaction increased during treatment with chemotherapeutic agents and ER-stress inducers. In CRC cells with SPARC overexpression, ER stress signaling was enhanced, as evidenced by an earlier activation of PERK/eIF2 α - and IRE1 α /XBP-1-mediated signaling following exposure to tunicamycin, 5-FU and CPT-11. Importantly, SPARC sensitizes CRC cells to ER stress signaling activation by interfering with the binding between GRP78 and ER stress sensors. Furthermore, GRP78 was found to counteract SPARC's pro-apoptotic effects in CRC under ER stress. This is supported by the observation that the relative expression level of GRP78 to SPARC was related to the level of chemosensitivity of CRC cells. In support of this observation, we also noted that tissues from CRC patients that demonstrated a higher GRP78:SPARC expression ratio had a lower overall survival. Taken together, these findings identify SPARC as a novel modulator of ER stress signaling, and further defines its role in enhancing chemotherapeutic response in CRC.

5.1.2 Chapter 4

In this Chapter, we demonstrated that HSP47 promotes cellular survival and reduces drug sensitivity in CRC cells by enhancing the activation of Akt signaling in CRC cells. We showed that the expression of HSP47 is significantly elevated in human CRC tissues, and individuals with elevated HSP47 expression tend to have poorer overall survival. This may be as a result of

HSP47's ability to suppresses apoptosis in CRC cells despite exposure to 5-FU. We also found that in HSP47-overexpressing CRC cells, there was greater and more prolonged activation of Akt when cells were exposed to chemotherapy. Conversely, Akt activity was subdued in knockdown cells with reduced HSP47 expression. Interestingly, we were able to demonstrate that the mechanism behind this persistent activation of Akt in CRC cells with elevated HSP47 expression may lie with this protein's ability to interact and destabilize PHLPP1, a phosphatase that dephosphorylates Akt at serine 473. In the presence of reduced PHLPP1 and thereby, reduced phosphatase activity, Akt retains its phosphorylated state at serine 473, which in turn promotes cellular survival even in the presence of environmental stressors (chemotherapy).

5.2 Conclusions Regarding the Study Hypotheses

In conclusion, the findings in both endogenous SPARC-expressing cells, as well as ectopic SPARC-overexpressing cell line, support our first hypothesis that SPARC increases chemosensitivity of CRC cells by interfering with the ER stress signaling via its interaction with GRP78. This current study validated the interaction between SPARC and GRP78. Furthermore, it demonstrated that SPARC interferes with the binding between GRP78 and ER stress sensors, which likely results in the activation of ER stress-associated cell death in CRC cells under chemotherapy.

In the second section of the thesis, the role of HSP47 in promoting drug resistance in CRC cells was identified. HSP47 was found to facilitate the activation of Akt signaling likely via its interaction with PHLPP1 in CRC cells following chemotherapy *in vitro* and *in vivo*. This supports our second hypothesis concerning HSP47 being involved in the modulation of chemotherapeutic response in CRC.

5.3 Strengths and Limitations

In the first section of the thesis, a mechanism by which SPARC suppresses the survival of CRC cells following chemotherapy was demonstrated. To my knowledge, it is the first time SPARC was identified to interact with GRP78 and mediate both PERK-eIF2 α and IRE1 α -XBP-1 stress signaling. Notably, this finding was observed in cell lines exposed to two main CRC therapeutic drugs 5-FU and CPT-11, implying a clinical relevance in this finding. Moreover, a detailed mechanism explaining how SPARC modulates the stress signaling was also uncovered in this study. SPARC sensitizes ER stress signaling activation through its interference of the binding between GRP78 and the ER stress sensors. In addition, the clinical application can also be found by a new biomarker proposed (the relative expression level of GRP78 to SPARC) in this study to predict CRC chemosensitivity and a patient's prognosis.

However, the primary limitation of this study is the lack of an *in vivo* xenograft model using cell lines with different GRP78 to SPARC expression level to demonstrate the effects of GRP78-SPARC interaction on the chemosensitivity of CRC cells. Nevertheless, as the focus of this study was to unravel the molecular mechanisms by which SPARC modulates the signaling response in CRC following chemotherapy, the lack of animal study, although rendering the work less complete, does not influence the validity of the findings that SPARC mediates ER stress signaling via its interaction with GRP78.

Another limitation of this study is the lack of validation of the proposed biomarker in a large cohort such as TCGA. The clinical samples collected in most online databases are from bulk tumors which are usually contaminated with stroma. As SPARC is highly expressed in fibroblasts in the stroma, expression analysis with RNA-seq or microarray data may not represent the actual SPARC expression level in the tumors. Although the analysis of our TMA,

which contains more than one hundred samples, shows an association between high GRP78:SPARC ratio with poor prognosis, validation of these results with a larger cohort will still be needed to confirm the validity of the proposed biomarker.

For the second section of the thesis, HSP47 was identified as a potential novel target for CRC therapy and its involvement in Akt signaling is also a new finding. The elevated expression of HSP47 in CRC was observed in our paired clinical samples as well as two large cohorts (TCGA and curatedCRCData), providing a strong translational relevance of our findings. Furthermore, the role of HSP47 in promoting drug resistance was demonstrated with multiple cell lines to confirm the generality of the results. Opposite findings were also observed in both knockdown (loss-of-function) and overexpressing (gain-of-function) cell lines, strengthening the validity of this study. In addition, the *in vivo* confirmation of the results *in vitro* by using a xenograft model derived from overexpressing cell lines was also provided.

One of the limitations in this study is that we did not examine HSP47 protein expression level in CRC patients with tissue microarray. Nevertheless, the staining results in The Human Protein Atlas supports our findings that HSP47 protein expression is elevated in the majority of CRC tumors. Another limitation of this study is that we did not inhibit HSP47 and examine the subsequent effects on cell survival in the xenograft tumors of CRC patients. Instead, we validated the effects of HSP47 *in vitro* and *in vivo* by using the cell line model to avoid the influence of tumor heterogeneity. Future studies will help to substantiate that HSP47 is involved in the modulation of chemotherapeutic response in CRC.

5.4 Overall Significance and Clinical Implications

In the first section of this thesis, a novel mechanism by which SPARC promotes ER stress-associated death via its interaction with GRP78 was demonstrated. This suggests a combinatorial therapy using SPARC mimetics with current CRC chemotherapeutic drugs may reduce drug resistance and promote the treatment efficacy. In addition, we also found the relative expression level of GRP78 to SPARC is associated with the overall survival of CRC patients, suggesting it as a potential prognostic marker for CRC.

In the second section, HSP47 was firstly found to be related to chemoresistance in CRC and its expression level is associated with patient survival. This suggests HSP47 is a potential therapeutic target to increase treatment efficacy in CRC. Moreover, a novel mechanism by which HSP47 modulates Akt signaling was also identified, providing the rationale for the design of HSP47-target therapy in the future.

5.5 Future Research Directions

Given the strong evidence showing that SPARC promotes ER stress-associated death through its interaction with GRP78, it is important to further characterize the interaction between these two proteins in order to delicately modulate the ER stress signaling. First, the protein interaction in living cells can be further confirmed with yeast two-hybrid assay and BioID analysis. Isothermal titration calorimetry (ITC) assay can also be conducted to understand the binding affinity, stoichiometry, and thermodynamic parameters of these two proteins.

Following the confirmation of direct binding between GRP78 and SPARC in living cells, identification of the specific binding domains between these two proteins will need to be performed to increase our understanding of their functional relationship and facilitate SPARC-

based therapeutic drug design. To identify the binding domains of these two proteins, serial truncated proteins of GRP78 and SPARC will be constructed into affinity tagged expression vectors. These constructs will be transfected into HEK-293T cells and Co-IP assays will be performed to identify the mutual binding domains. The binding domain of SPARC to GRP78 will be used to design multiple partially overlapping peptides for peptide array synthesis in order to identify the peptides with the highest GRP78-binding affinity. The identified SPARC peptides will be tested for their ability to sensitize CRC cells to chemotherapeutic drugs and ER stress *in vitro* and *in vivo*.

For the second part of the thesis, as HSP47 was originally identified as a SPARC-binding protein at the initial stage of this study, the next question we are interested to know is whether and how SPARC participates in modulating the oncogenic function of HSP47. Validation of the interaction between SPARC and HSP47 will need to be performed, and the SPARC's effects on Akt signaling will also need to be examined. One of the questions that remain unanswered in this study is whether HSP47 is also able to interact with PHLPP2 and reduce its protein stability as it does with PHLPP1. As PHLPP1 and PHLPP2 target different Akt isoforms, it is important to understand whether there is an interaction between HSP47 and PHLPP2 in order to understand its complete effects on Akt signaling (44). In addition, as mentioned in Section 5.3, investigating the effects of HSP47 inhibition on chemotherapeutic response with the PDX model will strengthen HSP47's role as a therapeutic target in CRC. In addition to the establishment of CRC PDX model, tumors collected before and after treatment can be further characterized using massive parallel RNA sequencing and ingenuity pathway analysis to uncover unknown signaling events modulated by HSP47 in CRC. Moreover, as the study in this thesis is an initial attempt to understand the role that HSP47 has in CRC, only 5-FU was used in the experiments testing its

effects on chemoresistance. Other therapeutic drugs commonly used in CRC treatment, should also be included in future studies to test whether there is a synergistic effect of HSP47 inhibition and the cytotoxicity from other therapeutic agents. Targeting HSP47 is expected to enhance the potency of cetuximab and panitumumab as the two drugs exert their anti-tumor function mainly through inhibiting Akt signaling via their binding to EGFR (226)

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