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## INVESTIGATING THE ROLE OF PRMT1 AND ARGININE METHYLATION OF HSP70 IN HUMAN PANCREATIC CANCER

Liang Wang

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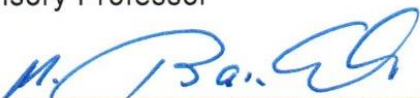
by

Liang Wang, B.S.

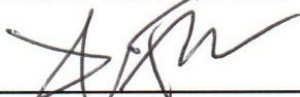
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**INVESTIGATING THE ROLE OF PRMT1 AND ARGININE METHYLATION OF HSP70  
IN HUMAN PANCREATIC CANCER**

A

DISSERTATION

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Liang Wang B.S.

Houston, Texas

August, 2017

## **DEDICATION**

I dedicate this dissertation to my parents. They taught me the qualities to be a good person and gave me courage and confidence to face any difficulty in my life. They were always supportive for my education in the past twenty years. This dissertation is dedicated to them for all the love they gave me.

## ACKNOWLEDGEMENTS

Many people have contributed to this dissertation and my colorful graduate school life in the past five years. Thus, first I want to give my sincere gratitude to all the people who have made this dissertation completed and my study experience at UT GSBS/MD Anderson Cancer Center so memorable in my life.

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I would like to thank my advisory committee members, Drs. Menashe Bar-Eli, Zhen Fan, Jian Gu, Craig Logsdon and Shao-Cong Sun, for their constructive suggestions and instructive questions during each of my committee meetings. Every member evaluated my study and gave me valuable feedback based on their own background and specialty. The discussions, sometimes debates, with my advisory committee, trained my scientific thinking, expanded my scientific knowledge and improved my presentation and writing skills. All of these lessens constitute an indispensable part of my graduate education.

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In the past five years, the graduate school and Cancer Biology Program were always supportive to my education. The courses I took pushed my knowledge and my study to a higher level. The Cancer Biology Program Retreat held every year was an exciting platform for students to share their ideas and personal stories. Many old and new friends made my life at Houston to be a good memory. My thanks go to all of them.

Last but not least, I must thank my parents for their endless love and encouragement. Although we lived apart in two countries during my study in the United States, my parents have been the constant source of love, care and strength for me all the time. The merits they passed to me will influence my entire life no matter what position I take and what place I go in the future. At this moment, I give my heartfelt thanks to my parents for their trust and love to me.

# **INVESTIGATING THE ROLE OF PRMT1 AND ARGININE METHYLATION OF HSP70 IN HUMAN PANCREATIC CANCER**

Liang Wang B.S.

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Protein arginine methyltransferase 1 (PRMT1) is the major arginine methyltransferase, which catalyzes the addition of one or two methyl groups to the arginine residues of its substrate proteins. The best known substrate for PRMT1 is histone, while more and more non-histone proteins are now found to be methylated by PRMT1. Dysregulation of PRMT1 is reported in several human cancer types. However, its biological roles in human pancreatic cancer initiation and development are still unclear. In the first part of this study, I found that the expression level of PRMT1 was elevated in both human and mouse pancreatic cancer tissues in immunohistochemistry analysis. The further functional studies demonstrated a pro-tumorigenic role of PRMT1 in several pancreatic cancer cell lines and mouse models.

In the second part of this study, several heat shock protein 70 (HSP70) family members were found to interact with PRMT1 in co-immunoprecipitation assays. Furthermore, using *in vitro* methylation assay and mass spectrometry analysis, I revealed that HSP70 was a novel methylation substrate of PRMT1, and two conserved arginine residues were identified as the methylation sites. By using HSP70 knockout cells generated by CRISPR/Cas9 system and a series of wildtype and methylation sites mutant HSP70 expression vectors, I demonstrated that PRMT1-mediated arginine methylation was essential for the drug resistance function of HSP70 in pancreatic cancer cells.

Collectively, my results not only elucidate the important role of PRMT1 in pancreatic cancer pathogenesis, but also suggest a novel underlying mechanism of HSP70-mediated

drug resistance to chemotherapeutic agents. Therefore, targeting PRMT1-HSP70 axis could be a new therapeutic regimen for pancreatic cancer.



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## ABBREVIATIONS

PDAC	pancreatic ductal adenocarcinoma
PanIN	pancreatic intraepithelial neoplasia
IPMN	intraductal papillary mucinous neoplasm
MCN	mucinous cystic neoplasm
PTM	post-translational modification
SAM	S-adenosyl methionine
MMA	monomethylarginine
ADMA	asymmetric dimethylarginine
SDMA	symmetric dimethylarginine
PRMT	protein arginine methyltransferase
GAR motif	glycine- and arginine-rich motif
PGM motif	proline-, glycine-, and methionine-rich motif
SPF30	splicing factor 30
TDRD	Tudor domain-containing protein
H4R3	arginine 3 of histone 4
ZEB1	zinc finger E-box-binding homeobox 1
EMT	epithelial-mesenchymal transition
53BP1	p53 binding protein 1
ER	estrogen receptor
FOXO1	forkhead box O1
BAD	BCL-2 antagonist of cell death
HSP	heat shock protein
ROS	reactive oxygen species
GRP	glucose-regulated protein

HSF	heat shock factor
HSE	heat shock element
NBD	nucleotide-binding domain
SBD	substrate-binding domain
BAG-1	Bcl-2-associated athanogene 1
CHIP	C terminus of HSP70-interacting protein
TNF	tumor necrosis factor
Rb	retinoblastoma
AML	acute myeloid leukemia
MDS	myelodysplastic syndrome
IHC	immunohistochemistry
TMA	tissue microarray
HRP	horseradish peroxidase
DAB	diaminobenzidine
HPSC	human pancreatic stellate cell
DMEM	Dulbecco's Modified Eagle's Medium
HBSS	Hank's balanced salt solution
IC50	half maximal inhibitory concentration
CCK-8	Cell Counting Kit-8
GI	gastrointestinal
HCC	human hepatocellular carcinoma
Co-IP	Co-immunoprecipitation
PMSF	phenylmethylsulfonyl fluoride
IPTG	isopropyl $\beta$ -D-1-thiogalactopyranoside
LSC	liquid scintillation counting
DPM	disintegrations per minute



PPI	protein-protein interaction
MEF	mouse embryonic fibroblast
GST	glutathione S-transferase
SOD2	superoxide dismutase 2
EGFR	epidermal growth factor receptor
MDR1	multidrug resistance protein 1

## **CHAPTER 1 BACKGROUND**

### **1.1 PANCREATIC CANCER**

The pancreas is a glandular organ belonging to both digestive system and endocrine system in human beings. It is located deep in the abdominal cavity and behind the stomach. The pancreas has two major functions that are performed by two functionally differentiated cell compartments. The exocrine part of pancreas, which consists of ductal and acinar cells and is about 80-90% of this organ, is responsible for producing pancreatic juice containing many digestive enzymes. These enzymes help to break down and absorb nutrient macromolecules in the small intestine. The endocrine part of pancreas is often called pancreatic islets, and is about 10-20% of this organ. The islets secrete several important hormones, such as insulin, glucagon, somatostatin, and pancreatic polypeptide, into the blood circulation to regulate the blood glucose level and maintain a proper metabolism within the body.

Pancreatic cancers have several subtypes and may occur in either exocrine or endocrine part of pancreas, but the former one, which is the typical pancreatic adenocarcinoma, is the most common form, and accounts for about 85% of all pancreatic cancer cases (1). The pancreatic endocrine tumors make up for less than 5% of all cases. Pancreatic cancer accounts for more than 200,000 deaths every year worldwide (2). In the United States, pancreatic cancer is now surpassing breast cancer and is the third leading cause of cancer-related death in the year 2016 (3). Moreover, it is expected to become the second leading cause of cancer-related death in the US by the year 2030, surpassing colorectal cancer (4).

Although the causes of pancreatic cancer are still insufficiently known, some risk factors have been identified. Age is the most important predictor of pancreatic cancer. The median age of diagnosis is 71 years and almost all patients are older than 45 years (1). Smoking,

diabetes mellitus, chronic pancreatitis, and obesity are known risk factors associated with pancreatic cancer (1). Consumption of red or processed meat, fat, and alcohol is also associated with increased risk while vegetarian diet is associated with decreased risk of pancreatic cancer (5).

It is estimated that 5 to 10 percent of pancreatic cancers are hereditary, but the precise genetic basis for familial aggregation has not been determined in most cases (6). Some established genetic disorders and affected genes that are known to increase the risk for pancreatic cancer include hereditary pancreatitis (*PRSS1*, *SPINK1*), familial atypical multiple mole and melanoma syndrome (*CDKN2A*), hereditary breast and ovarian cancer syndromes (*BRCA1*, *BRCA2*, *PALB2*), Peutz-Jeghers syndrome (*STK11*), hereditary nonpolyposis colon cancer (Lynch syndrome) (*MLH1*, *MSH2*, *MSH6*), Li-Fraumeni syndrome (*P53*), etc. (1, 7, 8). Although screening patients with an inherited predisposition for pancreatic cancer is of great value, there is currently no reliable screening methods to screen and detect pancreatic cancer early (5).

Because of the non-specificity and late appearance of symptom, most pancreatic cancer patients are diagnosed at advanced stages, which make them unsuitable for surgical resection of tumor tissues (5). According to an NCI cancer statistics review, the five-year survival rate in the US is around 8%, and median survival for pancreatic cancer patients is only 6 months (9). The dismal prognosis of pancreatic cancer is largely due to its late diagnosis, early metastasis, and resistance to conventional chemotherapy and radiotherapy.

Pancreatic ductal adenocarcinoma (PDAC) is by far the most common pancreatic cancer type. It is an invasive mucin-producing gland-forming neoplasm, and features of a very high rate of activating mutations in *KRAS* (>90%) and an extensive stromal reaction, which is termed desmoplasia (1). PDAC arises from non-invasive precursor lesions that are

microscopic non-invasive epithelial proliferations within the pancreatic ducts. The most prevalent type of such precursor lesions is named pancreatic intraepithelial neoplasias (PanINs)(1). Based on the architectural and cytological atypia, PanINs are categorized into low-grade PanIN1, intermediate-grade PanIN2, and high-grade PanIN3 dysplasia (1). Molecular pathology studies and genomic analyses have established a model of stepwise progression of PanIN from low grade to high grade and finally to invasive PDAC. During the long progression time of this disease (in human beings it can be 10 to 20 years), various genetic alternations accumulate at different stages. There are four major gene mutations, one oncogene and three tumor suppressor genes, that are usually regarded as driver mutations in PDAC (10). *KRAS* gene encodes a small GTPase that mediates multiple downstream signaling pathways from growth factor receptors, including PI3K-AKT pathway and Raf-MEK-ERK pathway. Activating mutation (most commonly codon 12 and 13) of *KRAS* renders its GTPase activity constitutively active, and is the most frequent and first somatic mutation that can be detected in PDAC progression (10). It occurs in about 40% of low-grade PanIN1 lesions and more than 90% in high-grade PanIN2 or 3 and PDACs. *CDKN2A* (also known as cyclin-dependent kinase Inhibitor 2A), which encodes an essential cell-cycle arrest regulator, is mutated in a loss-of-function way at relatively early PanIN stages (10). In PDAC, its mutation rate is more than 50%. By contrast, somatic mutations in *TP53* and *SMAD4* tumor suppressor genes often occur in PanIN3 and invasive carcinoma (10). Aberrant expression of p53 mutant proteins impairs multiple cellular stress responses, such as DNA damage repair and cellular senescence, while loss-of-function mutation of *SMAD4* disrupts normal TGF- $\beta$  signaling pathway (10). All these mutations further promote cancer progression to the advanced stage and/or tumor metastasis.

Besides PanIN, some PDACs arise from macroscopic cystic precursors—namely, intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs), although these two types are far less common than PanIN (2). IPMNs and MCNs are also categorized into low-grade, intermediate-grade, and high-grade dysplasia based on the degree of dysplasia in the lining epithelium, but the genetic alternations occurring in these precursor lesions are not the same as PanINs. For example, mutations in the oncogenic *GNAS* gene occur only in IPMNs, whereas inactivating mutations in the ubiquitin ligase *RNF43* occur in both IPMNs and MCNs (11, 12).

In addition to PDAC, there are some other types of pancreatic tumors that belong to pancreatic exocrine tumor, such as acinar cell carcinoma, pancreatoblastoma, solid-pseudopapillary carcinoma, serous cystadenocarcinoma, intraductal papillary-mucinous tumor, mucinous cystadenoma, osteoclast-like giant cell tumor, miscellaneous carcinoma and so on (10). Pancreatic cancer derived from endocrine cells are less than 5 percent of all primary pancreatic tumors, and most of the pancreatic endocrine tumors occur in aged people. It mainly includes insulinoma, small cell carcinoma, glucagonoma, gastrinoma and so on (13). Non-epithelial tumors have two types, soft tissue tumor and lymphoma. This dissertation studied the molecular mechanisms of biology and pathogenesis of pancreatic ductal adenocarcinoma, and potential therapeutics for this disease. Thus the term pancreatic cancer in this dissertation is referred to pancreatic ductal adenocarcinoma without specific explanation after this point.

To date, the clinical available treatments for pancreatic cancer are still very limited. Surgical resection is the only way that can cure this disease and significantly increase survival time compared with other treatment options, but only 15-20% of pancreatic cancer cases are resectable at the time of diagnosis (1). Chemotherapy is the mainstay of treatment for locally advanced and metastatic pancreatic cancer. Gemcitabine, a

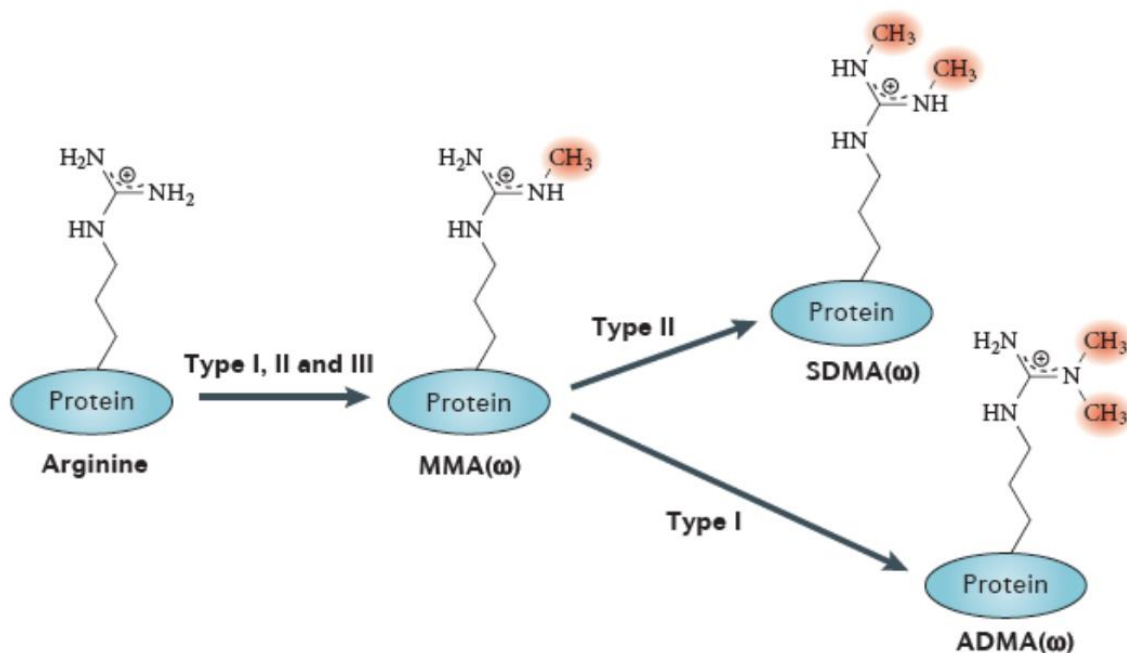
nucleoside analog, is the only widely accepted first-line chemotherapeutic agent for pancreatic cancer in the past two decades (2). However, a high degree of intrinsic and acquired resistance to gemcitabine is often observed in clinical practice, which limits the efficiency of this chemotherapy (14). From a mechanistic perspective, gemcitabine resistance may result from alterations in genes/proteins involved in drug transportation and metabolism, or from the influences of cancer microenvironment. To understand, monitor and overcome the drug resistance against gemcitabine will benefit pancreatic cancer patients profoundly.

Two recent clinical trials compared combination chemotherapy over single-agent gemcitabine to treat pancreatic cancer. In these studies, combined use of fluorouracil, irinotecan, oxaliplatin, and leucovorin (FOLFIRINOX), or gemcitabine plus albumin bound paclitaxel particles (nab-paclitaxel), improved the survival and quality of life of patients with metastatic pancreatic cancer (15, 16). Results from these studies may provide new directions for pancreatic cancer chemotherapy in the future. Mutant KRAS targeting drug has been a hot study focus for decades. However, due to the undruggable nature of mutant KRAS protein, small molecule drug targeting mutant KRAS are still not available in clinical use. To bypass the KRAS obstacle, researchers put many efforts on investigation of other types of alterations involved in pancreatic cancer pathogenesis, including epigenetic, transcriptional, and proteomic alterations. Among them, the post-translational modification (PTM) of vital proteins is believed to be an important way to control protein expression and/or functions in a highly dynamic mode to help tumor cells adapt to and survive the harsh microenvironment. The studies on PTM regulation will not only expand our knowledge on tumor initiation and progression, but may also reveal additional targets for novel approaches in cancer diagnosis and therapy.

## **1.2 PROTEIN ARGININE METHYLATION AND PROTEIN ARGININE METHYLTRANSFERASE**

Post-translational modification is the process to add one or more chemical groups to a protein after its translation. Common types of PTMs are phosphorylation, acetylation, ubiquitination and methylation, etc. By attaching and removing different chemical groups to and from specific amino acid residues in a protein, the three dimensional conformation, positive or negative charges, hydrophilic or hydrophobic property of this protein is quickly changed, and subsequently, the protein stability, subcellular localization and/or its interacting molecules are altered correspondingly. PTMs give cells, particularly cancer cells, the ability to quickly and efficiently regulate the functions of some vital proteins to help them deal with the ever-changing external and internal microenvironments.

Protein methylation is the process that one or more methyl groups are added to the nitrogen side chains in the arginine and/or lysine residues of proteins by S-adenosyl-methionine (SAM) dependent methyltransferases. Arginine is unique among the amino acids as its guanidino group contains five potential hydrogen bond donors that are positioned for favorable interactions with biological hydrogen bond acceptors (17). Each methyl group added to an arginine residue of a protein not only changes its conformation, but also removes a potential hydrogen bond donor, which may change the interacting repertoire of this protein and therefore affect its physiological functions. For example, arginine methylation of the Sam68 proline-rich motifs can inhibit its binding to SH3, but not WW domains (18). Importantly, methylation does not neutralize the cationic charge of an arginine residue (19).



**Figure 1. Three types of methylation on arginine residues.**

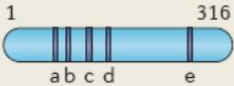
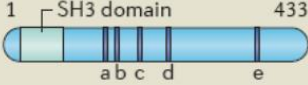
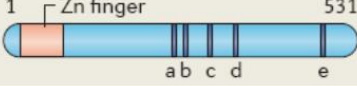
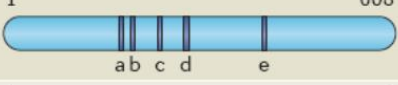



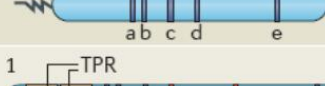

Types I, II and III protein arginine methyltransferases (PRMTs) generate mono-methylarginine (MMA) on one of the terminal ( $\omega$ ) guanidino nitrogen atoms. At this step, the two nitrogen atoms are equivalent. Subsequently, type I enzymes can catalyze the formation of asymmetric dimethylarginine (ADMA), while type II enzymes can catalyze the formation of symmetric dimethylarginine (SDMA). On certain substrates, PRMT7 functions as a type III enzyme, which only generates MMA products. To date, no enzyme has been found that forms both ADMA and SDMA modifications. *Adapted from Yang Y. and Bedford M., Nat Rev Cancer. 2013,13(1):37-50 with the permission from Nature Publishing Group.*



There are three types of arginine methylation that can be found in mammalian cells:  $\omega$ - $N^G$ -monomethylarginine (MMA),  $\omega$ - $N^G,N^G$ -asymmetric dimethylarginine (ADMA) and  $\omega$ - $N^G,N^G$ -symmetric dimethylarginine (SDMA) (Fig.1)(17). The formation of MMA, ADMA and SDMA in mammalian cells is carried out by a sequence-related family of nine protein arginine methyltransferases (PRMTs)(17)(Table 1). Each PRMT species harbors the characteristic motifs of seven beta strand methyltransferases (20), and all PRMTs can catalyze MMA formation. Additionally, Type I PRMTs (PRMT1, PRMT2, PRMT3, PRMT4/CARM1, PRMT6 and PRMT8) can further catalyze ADMA formation, in which the second methyl group is added to the same guanidino nitrogen atom, while Type II PRMTs (PRMT5 and PRMT9) can add the second methyl group to the other guanidino nitrogen atom of the arginine, resulting in an SDMA (17). Particularly, PRMT7 is the only Type III enzyme, exclusively catalyzing the formation of MMA (21, 22). Most of the PRMTs are ubiquitously expressed, except for PRMT8, which is reported to be predominantly expressed in the brain (23).

Most PRMTs methylate glycine- and arginine-rich (GAR) motifs in their substrate proteins. Indeed, PRMT1, PRMT3, PRMT6 and PRMT8 mainly recognize and methylate RG or RGG repeats in GAR motifs, but they do not seem to be functionally redundant *in vivo*, because cells knock-down or -out of these enzymes display different abnormalities, and differential proteins are hypomethylated in those cells (17). Some PRMTs have their own substrate specificity. For example, PRMT4/CARM1 preferentially modifies arginine residues present in proline-, glycine-, and methionine-rich (PGM) regions rather than GAR motifs. PRMT5 modifies substrates containing both GAR and PGM motifs, while

**Table 1: mammalian PRMT family members**

PRMT (locus)	Domain structures of human enzymes*	Function	Family
PRMT1 (19q13.3)		Transcription activation, signal transduction, RNA splicing and DNA repair	Type I
PRMT2 (21q22.3)		Transcription regulation	Type I
PRMT3 (11p15.1)		Ribosomal homeostasis	Type I
CARM1 (19p13.2)		Transcription activation, RNA splicing, cell cycle progression and DNA repair	Type I
PRMT5 (14q11.2)		Transcription repression, signal transduction and piRNA pathway	Type II
PRMT6 (1p13.3)		Transcription regulation	Type I
PRMT7 (16q22.1)		Male germline gene imprinting	Type II and type III
PRMT8 (12p13.3)		Brain-specific function	Type I
PRMT9 (4q31.23)		Unknown	Not classified

The catalytic methyltransferase domains contain a number of highly conserved motifs (vertical dark blue lines marked as a, b, c, d and e) that are important for the enzymatic reaction. Additional motifs: SH3 domain (SH3), zinc finger domain (ZnF), myristoylation motif (Myr), tetratricopeptide repeat (TPR). Red vertical lines indicate poor sequence similarity to the indicated PRMT motif. Adapted from Yang Y. and Bedford M., *Nat Rev Cancer*. 2013,13(1):37-50 with the permission from Nature Publishing Group.

PRMT7 preferentially modifies substrates with an RXR motif, consisting a pair of arginine residues separated by one basic residue (21). One thing should be emphasized is that the preferential motif for PRMTs is not a stringent rule, because many PRMT substrates have been found that they do not contain these typical motifs (24, 25). As a result of arginine methylation, GAR and PGM methylated motifs interact mainly with proteins via their Tudor domains (26). The human genome encodes over thirty members of these proteins, such as human survival motor neuron protein, splicing factor 30 (SPF30) and Tudor domain-containing proteins (TDRD) subfamily, which are functionally involved in many cellular processes (27).

PRMT1 and PRMT5 are the major asymmetric and symmetric arginine methyltransferases, respectively, and knockout either of these enzymes leads to early embryonic lethality in mouse models (28, 29). Other PRMTs have fewer substrates and have evolved more specialized cellular localization and/or functions. The aberrant expression of PRMTs and the dysregulation of their enzymatic activity have been associated with many diseases, including neurodegenerative diseases and several types of cancer (30, 31). Insights into distinct roles of different PRMTs are anticipated to provide new approaches for cancer prevention, diagnosis and treatment.

PRMT1 is the first mammalian PRMT identified as a single gene product, and is the primary PRMT that its activity accounts for more than 90% of the methylarginine residues in mammalian cells (32). PRMT1 has a very wide substrate specificity, and proteins localized in cell nucleus, cytoplasm and membrane can be methylated by PRMT1. Upregulation of this protein is observed in many types of human cancers, including breast, prostate, lung, colon, bladder cancer and leukemia (30). The best studied substrate for PRMT1 is histone. Methylation of arginine residues in histone tails is a part of the “histone code”, which is believed to be an important process of epigenetic regulation. Arginine 3 of

histone 4 (H4R3) is the primary site that PRMT1 specifically deposits an ADMA, which is a marker for transcription activation (30). In prostate cancer, the status of H4R3 methylation correlates with higher tumor grade and can be used to predict the risk of cancer recurrence (33). ZEB1 is a key transcription factor inducing the epithelial-mesenchymal transition (EMT) by repressing the transcription of E-cadherin. H4R3 methylation at the ZEB1 promoter activates its transcription and thereby promotes migration and invasion of breast cancer (34).

A large number of non-histone PRMT1 substrates that involved in multiple cellular processes have also been identified. BRCA1 has tumor suppressor activity in cell cycle regulation, DNA damage repair and chromatin remodeling. It was found to be methylated by PRMT1 in both breast cancer cell lines and tumor specimens (35). This methylation changes recruitment of BRCA1 to specific gene promoters, and dysregulation of BRCA1 methylation could result in genomic instability. Moreover, PRMT1 methylates the DNA repair pathway proteins MRE11 and p53 binding protein 1 (53BP1) (36, 37). Aberrant methylation of these proteins disrupts their proper localization to damaged DNA and prevents the recruitment of DNA repair machinery, which leads to an accumulation of DNA damage. PRMT1 also methylates some proteins that are involved in cell signaling transduction. For instance, in estrogen signaling, the estrogen receptor  $\alpha$  (ER $\alpha$ ) is methylated by PRMT1 at arginine 260 (R260) in the DNA binding domain, and estrogen treatment of MCF7 cells rapidly increases R260 methylation (38). This modification promotes the formation of a protein complex consisting of methylated ER $\alpha$ /Src/ PI3K, and the subsequent activation of AKT pathway. High level expression of methylated ER $\alpha$  and this protein complex is also an independent marker of poor prognosis of breast cancer (39, 40).

PRMT1-mediated methylation is found to crosstalk with other PTMs adjacent to the methylated arginine residues. One such example is that methylation of the Forkhead box O1 (FOXO1) transcription factor, on R248 and R252 residues in an AKT consensus phosphorylation motif, can block AKT-mediated phosphorylation on adjacent S253 residue, and therefore prevent FOXO1 proteasomal degradation and increase its nuclear localization and transcriptional activity on its targeting genes (41). Likewise, a crosstalk between PRMT1-mediated methylation and AKT-mediated phosphorylation is observed in the BCL-2 antagonist of cell death (BAD) protein, and affects BAD function in apoptosis regulation (42).

### **1.3 HEAT SHOCK PROTEINS AND CANCER**

Heat shock protein (HSP) is a large group of proteins involved in protein folding and maturation. Their expression is induced by heat shock or other environmental stimuli. Traditionally, HSPs are also known as molecular chaperones due to their physiological and protective roles in cells. They facilitate protein folding in protein synthesis and maturation process, and prevent aggregation of proteins when cells are exposed to environmental challenges, such as high temperature, hypoxia, heavy metals toxicity, reactive oxygen species (ROS), drugs, or other chemical agents that may induce cell stress or protein denaturation (43). HSPs are usually classified based on their molecular weights and include HSP27, HSP40, HSP60, HSP70, HSP90, and large HSPs (HSP110 and glucose-regulated protein 170, GRP170). Except for the group of small HSPs, like HSP27, HSP proteins have ATPase activity, and their molecular chaperone function is ATP-dependent. Heat shock factors (HSFs) are transcription factors that can bind to the heat shock elements (HSEs) upstream of HSP genes to induce the expression of HSPs when cells are exposed to various stresses (44).

HSPs are highly conserved proteins that they are found in virtually all living organisms, from bacteria to humans, which substantiate their important roles in life. During long time of evolution, HSPs evolved into many functionally related proteins, but their tissue specificity, subcellular localization and the stresses they deal with are distinct from each other. In this dissertation, the function and post-translational modification of HSP70, as well as some other members in this family, are studied. Therefore, some background knowledge of HSP70 protein family is introduced in this section.

The HSP70 family is encoded by the *HSPA* gene family and consists of 13 members in mammals (45). HSP70 proteins have a highly conserved domain structure, including the 44 kDa N-terminal nucleotide-binding domain (NBD), which has ATPase activity, the 18 kDa substrate-binding domain (SBD), and the 10 kDa C-terminal lid domain. When the NBD domain is ADP- or ATP-bound, the lid domain is in a closed or open conformation, which facilitate the binding or release of client proteins (46). HSP70 chaperone activity is regulated by some co-chaperones, such as HSP40, Bcl-2-associated athanogene 1 (BAG-1), and C terminus of HSP70-interacting protein (CHIP) (43).

There are five important members in HSP70 family that are well-studied and shown to be associated with cancer (43). The HSP70 (also known as HSPA1 or HSP72, ~72 kDa) and HSP70B' (also known as HSPA6, ~71 kDa) are stress-inducible protein chaperons, while HSC70 (also known as HSPA8, ~73 kDa), GRP75 (also known as HSPA9 or mortalin, ~75 kDa), and GRP78 (also known as HSPA5 or BIP, ~78 kDa) are constitutively expressed in cells. HSP70, HSP70B' and HSC70 are mainly localized in the cytosol, while GRP75 and GRP78 are mainly localized in mitochondria and endoplasmic reticulum (ER), respectively. All these HSP70 proteins have crucial functions in facilitating protein folding, maintaining the natural conformation of their client proteins, and promoting cell survival following diverse stresses. The role and dysregulation of these proteins in cancer

development is a hot topic in cancer research and has been reported by several studies (43, 47).

HSP70 is reported to be overexpressed in multiple tumor types, including colon, liver, prostate, esophagus and cervix. Later on, the prognostic significance of HSP70 has been proved in several human cancers, and it is independent on other prognostic factors (48). Although HSP70 is overexpressed in most human cancers, lower expression of HSP70 is observed in certain cancers, such as renal cancer (49). Therefore, by now HSP70 has not been accepted as a clinical biomarker of any cancer parameter.

Observations that many types of tumors have elevated levels of HSP70 indicate that HSP70 may have a pro-survival function in tumor cells. Since tumor cells live under conditions of continuous stress, such as hypoxia, nutrient deprivation, low pH and/or ROS, survival and development of tumor require adaptations that overcome stress-induced apoptosis. Indeed, HSP70 was discovered to suppress apoptosis by inhibiting both intrinsic and extrinsic apoptotic pathways (47). Besides, HSP70 was also shown to be able to inhibit oncogene-induced senescence and stabilize lysosome membrane that allows for autophagy (47). Because of these functions, HSP70 is required by tumor cells rather than normal cells for their survival and growth. Moreover, mutant HSP70 protein lacking chaperone function still retained their ability to protect cells from tumor necrosis factor (TNF)-induced apoptosis, indicating a non-chaperone function of HSP70 in cell biology (50).

HSP70 protein promotes tumorigenesis and it is also involved in mediating drug resistance in cancer therapy. For example, overexpression of HSP70 in fibrosarcoma WEHI-S cells increased their resistance against gemcitabine and topotecan (51). Knockdown of HSP70 enhanced the sensitivity to cisplatin in cervical cancer cells (52). Overexpression of HSP70 promoted bortezomib resistance, while inhibition of HSP70 enhanced

bortezomib-induced cell death in human bladder cancer cells (53). Given its important roles in cancer biology, HSP70 protein is a promising drug target that many drugs targeting this protein are under investigation either as monotherapy or as combined therapy with other chemo drugs. These drugs interrupt HSP70 functions via different mechanisms, such as blocking HSF1-dependent transcription of HSP70, decreasing ATPase activity of HSP70, disrupting the interaction of HSP70 with its co-chaperon BAG3, and so on (43).

Other members in HSP70 family are also reported to have cancer related functions. For example, HSC70 can bind to the non-phosphorylated tumor-suppressor retinoblastoma (Rb) protein and to mutant forms of p53 and p73 to inhibit their degradation (54); GRP75 is involved in myeloid malignancies and is commonly deleted in patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) (55); GRP78 is responsible for maintaining normal ER functions and prevent ER stress-induced apoptosis in cancer cells (56). GRP75 and GRP78 can also mediate drug resistance in cancer therapy, and they are reported be potential therapeutic targets for anticancer drugs (43). Inhibiting the expression and/or function of these proteins by siRNA, antibodies, natural compound or small molecule drugs can alleviate drug resistance in certain cancer cells (43).

Since most HSP70 proteins are stress-induced and elevated expressions of some members are observed in cancers, the regulation of HSP70 proteins was studied in the past two decades. Transcriptional regulation is the best known mechanism used by cells to increase HSPs under environmental stress. HSF-1 is considered to be the main heat shock factors (HSFs) that are responsible for transcriptional activation of *HSPA* genes. Under normal conditions, HSF-1 is retained by HSP70 and HSP90 in the cytoplasm as an inactive monomer. In response to stress, HSF-1 is released and a HSF-1 homotrimer is



formed that it binds to the heat shock elements (HSEs) upstream of *HSPA* promoters, thereby triggering *HSPA* gene transcription (57).

Apart from transcriptional regulation, HSP70 protein levels have also been found to be regulated at the post-transcriptional level either by micro-RNAs (miRNAs) or a recently identified mRNA post-transcriptional modification, N(6)-methyladenosine (58). Under heat shock condition, certain adenosines within the 5'UTR region of newly transcribed HSP70 mRNA are preferentially methylated to form N (6)-methyladenosine. This modification enables cap-independent mRNA translation initiation to rapidly increase HSP70 protein level within cells under heat shock condition (58).

Compared with above mentioned regulation of HSP70, little is known about the post-translational processing of HSP70 proteins. Although some amino acid residues are found to be phosphorylated, acetylated, malonylated or methylated, how they affect HSP70 protein function remains unclear (59). Phosphorylation of Ser400 of HSP70 is critical for the nuclear distribution of this chaperone (60). Methylation was first found to occur on a conserved lysine residue (K561) of HSP70, and the lysine trimethylation altered the affinity of this chaperone to its client protein,  $\alpha$ -synuclein (61). During my dissertation work, the first arginine methylation of HSP70 was reported that CARM1/PRMT4-mediated monomethylation of arginine 469 regulated HSP70 function in *RAR $\beta$ 2* gene activation (62). Since HSP70 is a highly dynamic protein and post-translational modification is an energy-efficient and quick way to modulate protein trafficking, function and/or its interactions with other partners, this dissertation will focus on the study of post-translational modification, mainly arginine methylation, of HSP70 protein.

## **CHAPTER 2 DEFINITIVE ROLE OF PRMT1 IN PANCREATIC CANCER PATHOGENESIS**

### **2.1 INTRODUCTION**

Pancreatic ductal adenocarcinoma (PDAC) is one of the most deadly malignancies. Although some driver oncogenes, like *KRAS*, have been identified and characterized, the molecular mechanisms underlying PDAC carcinogenesis, particularly the early initiation stage, are still not well understood. Moreover, given that PDAC is less sensitive to the conventional chemotherapy and *KRAS* is believed to be undruggable, the discovery of novel molecular targets for PDAC prevention and/or intervention is urgently needed.

As it is outlined in Chapter 1, protein arginine methylation, as one of the common post-translational modifications (PTMs), plays important roles in many biological processes, such as gene transcription regulation, RNA processing, signal transduction and protein trafficking, etc., and aberrant expression of some PRMTs are associated with many human cancers (30). However, since methyl group is the smallest chemical group, of which the molecular mass is only 14, that can be added to a protein, and its addition does not change the electric charge of the protein, protein methylation is more difficult to detect and study when compared with other PTMs, like phosphorylation. Therefore, protein arginine methylation is generally less studied except for histone methylation. Before the beginning of my dissertation study, the expression and activity of PRMTs and the protein arginine methylation status in PDAC have not been reported yet. Some studies showing the functions of PRMTs in other cancer types prompt us to study the roles of PRMTs in PDAC pathogenesis.

In this chapter, the expression pattern of some PRMTs, primarily PRMT1, in both human and mouse pancreatic cancer specimens was studied by immunohistochemical (IHC) staining. The relationship between its expression and some clinicopathologic parameters

was analyzed using a human pancreatic cancer tissue microarray. The effects of loss-of-function, either by siRNA or small molecule inhibitors, and gain-of-function of PRMT1 on cancer cell growth, migration and invasion *in vitro*, and tumor growth *in vivo* were studied. My study reveals that PRMT1 has tumor promoting function and it is a protein biomarker of poor prognosis for pancreatic cancer patients. Inhibition of PRMT1 expression or methyltransferase enzymatic activity can decrease tumor growth *in vitro* and *in vivo*.

## **2.2 MATERIAL AND METHODS**

### **Plasmids, siRNAs, antibodies and compounds**

Full-length PRMT1 transcript variant 1 plasmid was purchased from OriGene Technologies, and the ORF was subcloned into a pCMV6-AC-HA-His vector backbone with C-terminal HA-His tag. siRNA specifically targeting PRMT1 was purchased from Santa Cruze or synthesized by Sigma-Aldrich Corporate, with the sequence 5'-GCCAACAAGUUAGACCACG-3'. Cell transfection was performed with Lipofectamine 2000 transfection reagent according to manufacturer's suggestion (Thermo Fisher Scientific, Waltham, MA). Anti-PRMT1 (2449S) antibody was purchased from Cell Signaling Technology; anti-PRMT5 (07-405) antibody was purchased from EMD Millipore Corporation; and anti-HA (TA100012) antibody was purchased from OriGene Technologies. PRMT1 specific inhibitors DB75 and TC-E5003 were purchased from Tocris Bioscience.

### **Human and mouse tissue specimens and immunohistochemical staining**

Tissue microarray (TMA) or sections (5 µm thick) of formalin-fixed, paraffin-embedded human or mouse normal and tumor tissue specimens were prepared and processed for immunohistochemistry to detect various protein expressions using corresponding antibodies. Tissue slides were immersed in Dako target retrieval solution (Dako North America, Carpinteria, CA) and heated in a steamer for 30 minutes. Cool down slides to room temperature, and endogenous peroxidase was deactivated using 3% hydrogen peroxide in PBS for 10 min. All tissue specimens were covered by blocking solution, which is PBS (pH 7.4) containing 5% normal donkey serum and bovine serum albumin, and incubated for 30 minutes at room temperature. Tissue specimens were covered by primary antibodies (1:150-1:400 dilution in 5% BSA solution), and incubated overnight at 4°C. The samples were then rinsed by PBS for 3×5 minutes and incubated for 1 hour at room

temperature with Horseradish Peroxidase (HRP) conjugated secondary antibodies. Next, the slides were rinsed by PBS for 3×5 minutes and incubated with diaminobenzidine (DAB) for 2 to 10 minutes. The slides were washed three times with distilled water, counterstained with Harris hematoxylin (Fisher Scientific, Kalamazoo, MI), and washed in running tap water for 2 minutes. Finally, the slides were mounted using Permount Mounting Medium (Biomedica Corporation, Foster City, CA) and examined or photographed using a bright-field Leica microscope.

A positive signal was indicated by a reddish-brown color staining. The percentage of positive signal area was scored as (percentage scores) : <10% (0), 10–25% (1), 25–50% (2), 50–75% (3), and >75% (4). The intensity of staining was scored as (intensity scores): no staining (0), light brown (1), brown (2), and dark brown (3). The overall score is obtained by multiplying percentage score and intensity score. The overall score <4 is regarded as negative/weak expression,  $4 \leq \text{overall score} < 8$  is regarded as moderate expression and overall score  $\geq 8$  is regarded as strong expression. For each mouse sample, five or six randomly selected fields were scored and calculated to get the average staining score. All tissues sections were scored by 2 independent investigators prior not knowing the patient outcomes, and the mean values of 2 independent scores are presented.

### **Cell line information and cell culture conditions**

All human pancreatic adenocarcinoma cell lines and human embryonic kidney cell line 293T were purchased from the American Type Culture Collection (Manassas, VA) if it is not specified otherwise. FG human pancreatic adenocarcinoma cells were established by Vezeridis et al (63). Human pancreatic stellate cells (HPSC) were kindly provided by Rosa Hwang (64). All of the cell lines were maintained in plastic flasks in 37°C 5% CO<sub>2</sub> incubators as adherent monolayer in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential

amino acids, L-glutamine, penicillin/streptomycin and a vitamin solution (Flow Laboratories, Rockville, MD).

### **Animal information and animal care**

Female athymic nude mice and C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). The *Pdx1-Cre; LSL-Kras<sup>G12D</sup>* mice have been described previously (65). All mice were housed in laminar flow cabinets under specific pathogen-free conditions and were used when they were 8 weeks old. The animals were maintained in MD Anderson Cancer Center animal facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and National Institutes of Health.

### **Western blot analysis**

Cells were harvested at ~80 to 95% confluence and lysed with cell lysis buffer. Protein concentrations were measured using the Bradford Protein assay (Bio-Rad Laboratories). Lysates were boiled in LDS sample buffer (Thermo Fisher Scientific Inc.) for 10 minutes and cooled on ice for 5 minutes. Protein samples were separated in 10% SDS-PAGE gels for 2h in electrophoresis buffer (25 mmol/L Tris-HCl (pH 8.3), 192 mmol/L glycine, 0.1% SDS) and then electrophoretically transferred onto nitrocellulose membranes in transfer buffer (25 mmol/L Tris-HCl, 192 mmol/L glycine, 20% methanol) for 2 hour at 250 mA. The membranes were incubated in blocking buffer (5% nonfat milk in PBST) for 1 hour at room temperature with shaking and then rinsed twice briefly with PBST (PBS containing 0.1% Tween-20). The membranes were then incubated with primary antibodies diluted 1:1000 in %5 BSA solution at 4°C overnight. Then the membrane was washed three times with PBST and incubated with secondary antibodies (anti-rabbit or -mouse immunoglobulin,

horseradish peroxidase–linked F(ab)<sub>2</sub> fragment) diluted 1:2,000 in %5 BSA solution for 1 hour at room temperature with shaking. Targeting proteins were detected using enhanced chemiluminescence (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions.

### **Boyden chamber invasion assay**

Invasion chambers containing Matrigel-coated polyethylene terephthalate membranes with 8µm pores were purchased from BD Bio-Sciences in a 24-well plate format. Pancreatic cancer cells ( $3 \times 10^5$ ) in a 300-µL volume of serum-free medium were seeded in the upper compartments of invasion chambers. Ten percent fetal bovine serum medium was placed in the lower compartments as a chemoattractant, and invasion assays were carried out for 48 hours at 37°C. Each cell line or condition was tested in triplicate. To examine cell invasion, after 48 hour incubation, cells on the upper surface of the membrane were removed, and the cells invaded to the lower surface were fixed and stained with hematoxylin and eosin. Membranes were removed from the chambers, washed to remove excessive dye, and mounted on glass slides with lower surface facing up. Photos were taken under microscopy at 200x magnification and cell numbers were counted in 5 independent fields. Relative invasive activity was measured by calculating ratios of experimental cell numbers to control cell numbers.

### **Scratch wound healing assay**

Cells transfected with PRMT1 expression plasmid, pcDNA3.1 control plasmid or PRMT1 siRNA and control scramble siRNA were seeded in six-well plates until confluence. A wound was generated on the cell monolayer by scraping with a 10-µL pipette tip. Twelve hours later, the cells on the wounded monolayer were photographed, and cell migration was assessed by measuring wound sizes in multiple fields. There were no significantly

changes in cell numbers among those groups within 12h as were determined under a microscope in ten randomly selected fields at a magnification of 200×. Thus the impact of cell proliferation on “wound closing” ability was minimized within that time period.

### **Subcutaneous xenograft experiments**

Tumor cells ( $1 \times 10^6$ ) in 0.1 mL of Hank's balanced salt solution (HBSS) without calcium and magnesium were injected subcutaneously into the flanks of nude mice. The tumors' length and width were measured with a caliper twice a week. The tumor-bearing mice were sacrificed when they became moribund or on indicated time points after inoculation, and the tumors were removed and weighed. The tumor volumes ( $\text{mm}^3$ ) were calculated using the formula:  $\text{width}^2 \times \text{length} / 2$ . The administration of PRMT1 inhibitor, DB75, was intraperitoneal injected to the mice twice every week for three weeks. The low dose was 5mg/kg and high dose was 20mg/kg. Vehicle solution was used as control.

### ***In vitro* drug cytotoxicity assay**

Human PDAC cells were seeded in 96-well plates with 3,000 cells per well. PRMT1 inhibitors (DB75 and TC-E5003) were diluted in DMEM medium supplemented with 2% FBS. 200 $\mu$ l Medium with drug concentration ranging from  $10^{-7}$  to  $10^{-4}$  M or vehicle control were added to each well, and cells were cultured at 37°C for 72h. After incubation, cell viability was determined using Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Rockville, MD, USA), and IC<sub>50</sub> for each drug was calculated using Prism GraphPad 6 software. The viability of vehicle control group was determined as 100%, and each drug concentration was tested in triplicate.



**Statistics.**

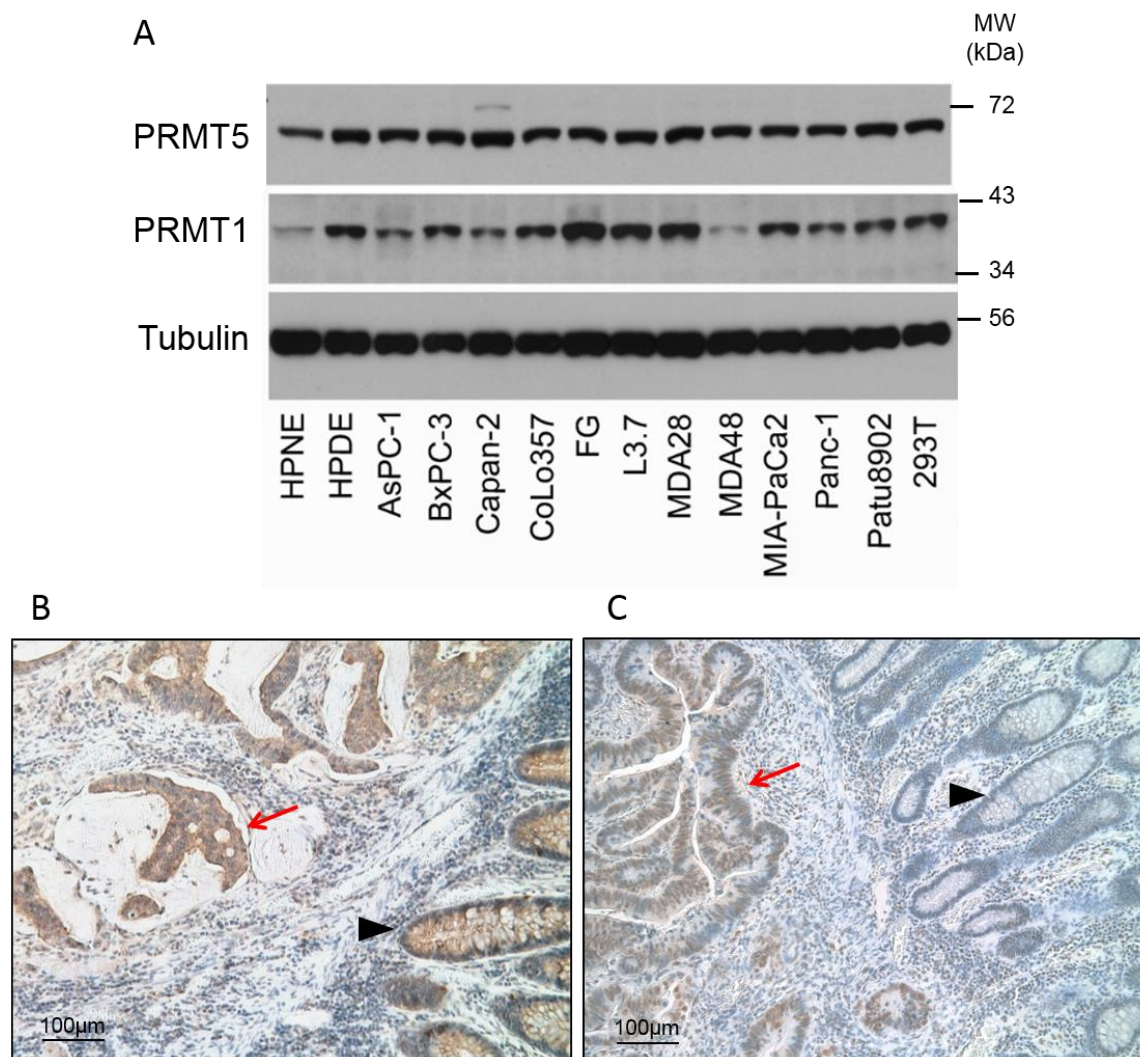
Kaplan-Meier survival curves were calculated using the overall survival time for each human patient. The log-rank test was used to test the significant differences between groups. For image quantification and staining score analysis, statistical significance was assayed by Student's *t*-test with Prism GraphPad software (two-tailed unpaired or paired *t*-test depending on the experiment—variance was first systematically examined using an *F*-test). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns: not significant. Data are represented as mean  $\pm$  SD or mean  $\pm$  s.e.m.

## **2.3 RESULTS**

### **2.3.1 PRMT1 is overexpressed in human PDAC cell lines and tumor tissues.**

There are nine PRMT members (PRMT1-9) encoded in mammalian genome, and they can catalyze the formation of three types of arginine methylation. Among nine PRMTs, PRMT1 and PRMT5 are the primary asymmetric (Type I) and symmetric (Type II) arginine methyltransferases, respectively. Thus the expression of these two proteins were first studied in human pancreatic cancer cell lines and colon tumor sections available in our laboratory (Fig.2). It is found that the expression levels of PRMT1 in a panel of PDAC cell lines were very different, from high expression, like FG, L3.7 and MDA 28 cells, to low expression, like AsPC-1, Capan-2 and MDA 48 cells (Fig.2A). In general, PRMT1 was highly expressed in PDAC cell lines when compared with immortalized human pancreatic duct cell line, HPNE. It is interesting to find that PRMT1 was moderately expressed in HPDE cells, which were generated by transduction of HPV16-E6, E7 genes (66). Thus the p53 and pRb pathways are inactivated in HPDE cell line. Whether this could be a reason for moderate PRMT1 expression awaits further study. In contrast to PRMT1, the expression level of PRMT5 is relatively homogeneous among all tested cell lines (Fig. 2A).

Consistent with the cell line data, the immunohistochemistry (IHC) staining results of human colon tumor sections also revealed that PRMT1 was overexpressed in tumor cells when compared with adjacent normal cells. The tumor cells showed stronger nuclear staining of PRMT1 than normal glandular epithelial cells (Fig.2C). However, there is no dramatic difference between the staining of PRMT5 in tumor and normal cells (Fig.2B). Based on these observations, I postulated that aberrant expression of PRMT1 was associated with cancer pathogenesis, and my following study would focus on the role of PRMT1 in carcinogenesis.



**Figure 2. PRMT1 is overexpressed in human PDAC cell lines and colon tumor tissues.**

**(A)** Western blot analysis of PRMT1 and PRMT5 expression in a panel of human PDAC cell lines, immortalized human pancreatic duct cell lines, HPNE and HPDE, and human embryonic kidney cell line, 293T.

**(B)** IHC staining of PRMT5 in colon tumor cells (red arrow) and normal cells (black arrow head).

**(C)** IHC staining of PRMT1 in colon tumor cells (red arrow) and normal cells (black arrow head).

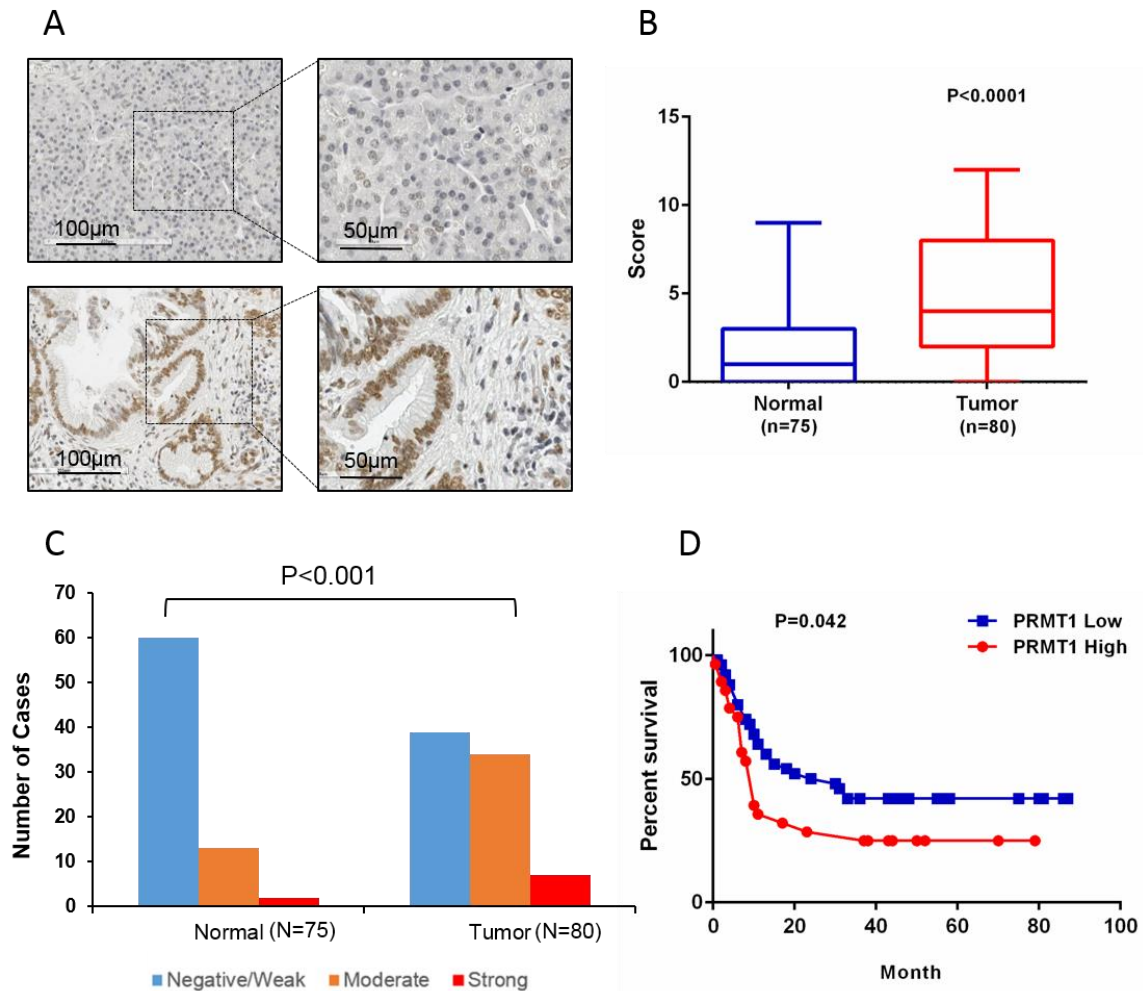
**Table 2. Clinicopathologic parameters and PRMT1 expression of the pancreatic cancer patients**

Variable	Total No.	PRMT1		P value
		High	Low	
<b>Age (n=76)</b>				0.670
<60	35	12	23	
≥60	41	16	25	
<b>Gender(n=77)</b>				0.367
Male	50	20	30	
Female	27	8	19	
<b>pT(n=76)</b>				0.543
T1	4	1	3	
T2	60	24	36	
T3	12	3	9	
<b>pN(n=77)</b>				0.310
N0	47	15	32	
N1	30	13	17	
<b>pM(n=77)</b>				0.129
M0	75	26	49	
M1	2	2	0	
<b>Histological Grade (n=77)</b>				0.029 *
1	7	3	4	
2	45	11	34	
3	25	14	11	
<b>pTMN(n=76)</b>				0.164
I	36	12	24	
II	38	14	24	
III	0	0	0	
IV	2	2	0	

\* A result is statistically significant when calculated p value is less than 0.05.

A human pancreatic cancer tissue microarray containing 90 paired tumor tissues and adjacent normal tissues was used to study the expression pattern of PRMT1. The patient information can be found in Table 2. In summary, elevated expression of PRMT1 was observed in tumor tissues than adjacent normal tissues, and PRMT1 was mainly expressed in the cell nucleus (Fig. 3A, B and C). High expression of PRMT1 was associated with shorter overall survival time of patients and poorer differentiation status of tumor tissues (Fig. 3D, Fig. 4 and Table 2).

To further validate the reactivity of PRMT1 antibody and generalize our observation in pancreatic cancer, human colon cancer and gastric cancer tissues were also IHC stained for PRMT1. Similarly, PRMT1 protein is overexpressed in tumor cells than adjacent normal epithelial cells, and it is mainly localized in cell nucleus (Fig. 5).



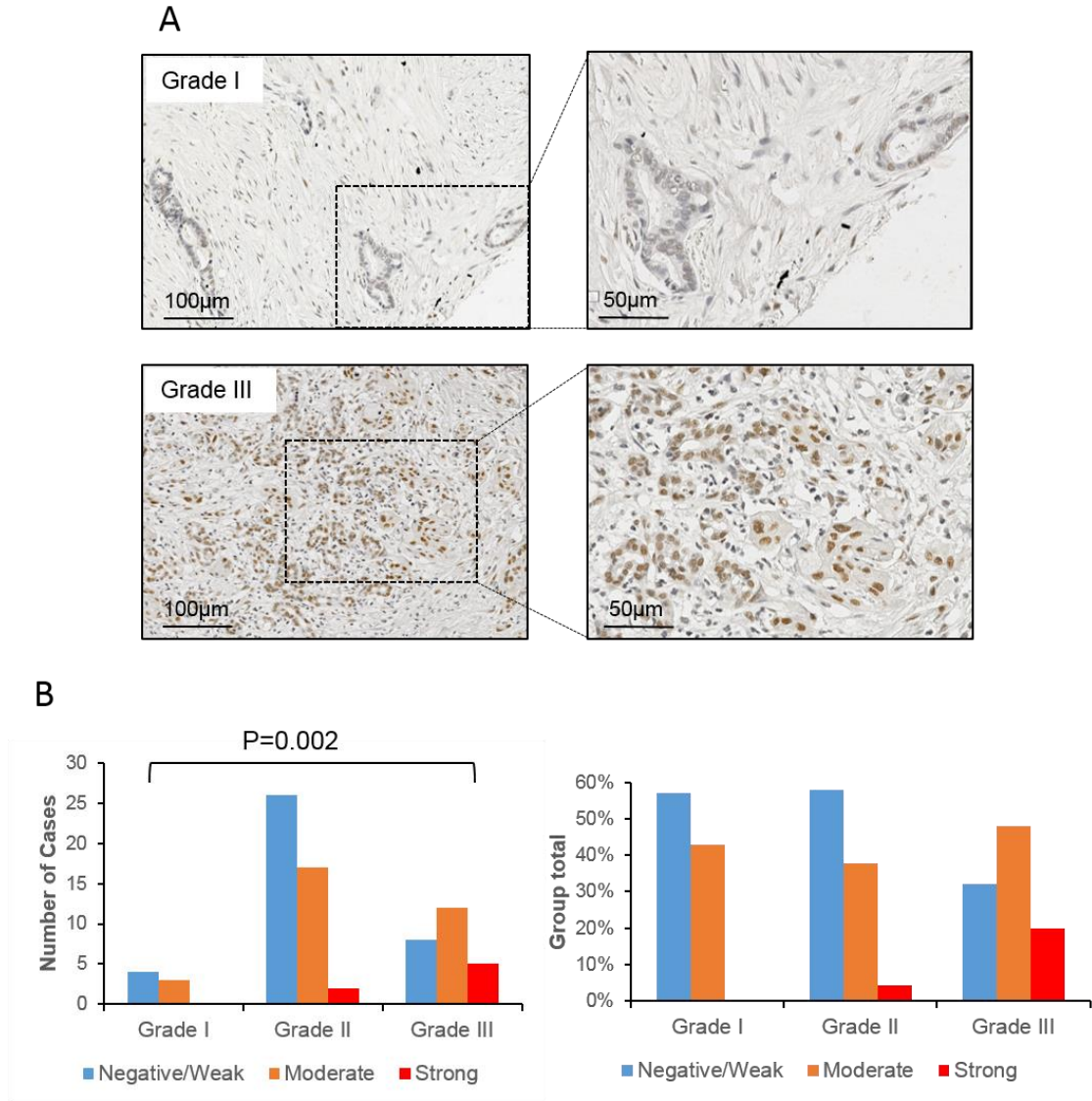
**Figure 3. PRMT1 is overexpressed in human PDAC tumor tissues and associated with shorter overall survival.**

A human pancreatic cancer tissue microarray was IHC stained with a specific anti-PRMT1 antibody.

**(A)** Representative images of PRMT1 expression in human PDAC tumor tissues (lower panel) and adjacent normal tissues (upper panel).

**(B and C)** Statistics of PRMT1 protein expression in human PDAC patients.

**(D)** Survival curve of PDAC patients with high (score $\geq$ 6) or low (score $<$ 6) expression of PRMT1 protein in tumor tissues.



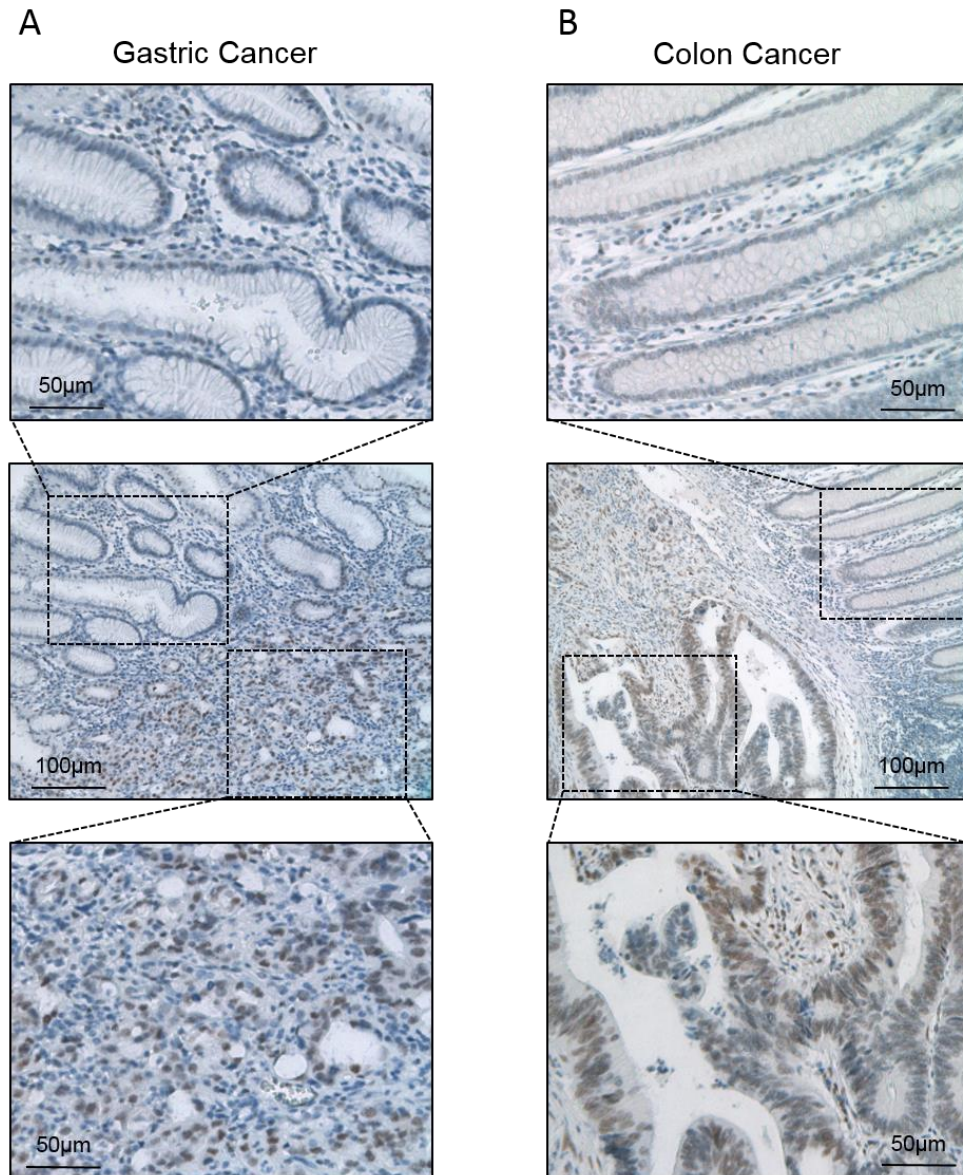
**Figure 4. Overexpression of PRMT1 is associated with high tumor differentiation grade.**

A human pancreatic cancer tissue microarray was IHC stained with a specific anti-PRMT1 antibody.

**(A)** Representative images of PRMT1 expression in human PDAC tumor tissues with differentiation grade I (upper panel) and grade III (lower panel).

**(B)** Statistics of PRMT1 protein expression in human PDAC patients with different tumor differentiation grades.





**Figure 5. Overexpression of PRMT1 in human gastric and colon tumor tissues.**

**(A)** Representative images of PRMT1 expression in human gastric tumor tissues (bottom panel) and adjacent normal tissues (top panel).

**(B)** Representative images of PRMT1 expression in human colon tumor tissues (bottom panel) and adjacent normal tissues (top panel).

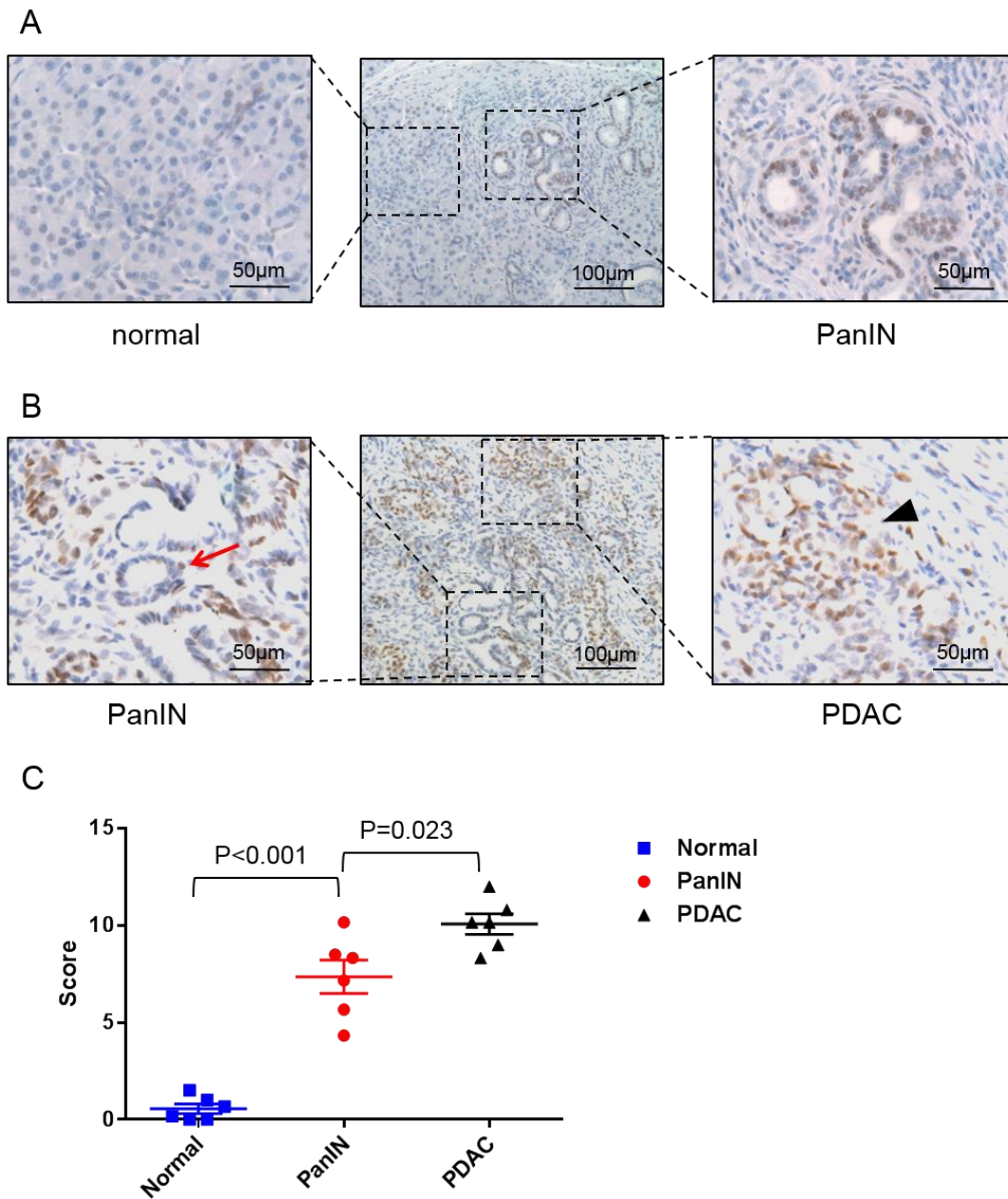


### 2.3.2 PRMT1 protein expression is gradually elevated during PDAC development in mouse models.

Since most human pancreatic cancer cases are diagnosed at relative late stages, it is difficult to observe pre-cancerous lesions, such as PanINs, in human patient pancreatic cancer specimens. Mutant *KRAS*-induced mouse pancreatic cancer model is widely used in preclinical researches, and these models recapitulate the step-wise development of pancreatic cancer seen in humans (67). The mouse line kept in our laboratory is the *LSL-Kras<sup>G12D/+</sup>; Pdx-Cre* model (also known as KC model). During mouse embryonic development, Cre recombinase is specifically expressed under the control of *PDX* promoter in all pancreatic cells that are derived from pan-pancreatic progenitor cells, and therefore *Kras<sup>G12D</sup>* is activated in mouse pancreas. By 8 weeks of age, the mice begin to develop early PanIN (PanIN 1A/B) lesions that slowly increase in both number and grade over the next 2 years. After a long progression time, usually > 8 months, a subset of these mice develop PDAC, with a median overall survival of 14 months (67). This mouse model gives us opportunities to observe pre-cancerous development at the initiation stage of mouse pancreatic cancer. When *p53<sup>R172H</sup>* mutation is crossed into the KC model, the *LSL-Kras<sup>G12D/+</sup>; p53<sup>R172H/+</sup>; Pdx-Cre* mice (also known as KPC mice) develop PanIN lesions on an accelerated schedule and will die of PDAC with a median survival of only 5.5 months (67). The KPC mice makes it possible to observe the entire process of mouse pancreatic cancer development in a relative short time period.

To study the expression alternation of PRMT1 during pancreatic cancer development, pancreatic tissue sections from both KC and KPC mice at various ages were IHC stained with a specific anti-PRMT1 antibody. I observed a positive staining of PRMT1 in both PanIN lesions and invasive PDAC lesions but no or very weak staining in mouse normal pancreas acinal cells (Fig.6). Moreover, the expression levels of PRMT1 were gradually

increased as disease developed from low grade PanIN to high grade PanIN and finally PDAC (Fig.7).

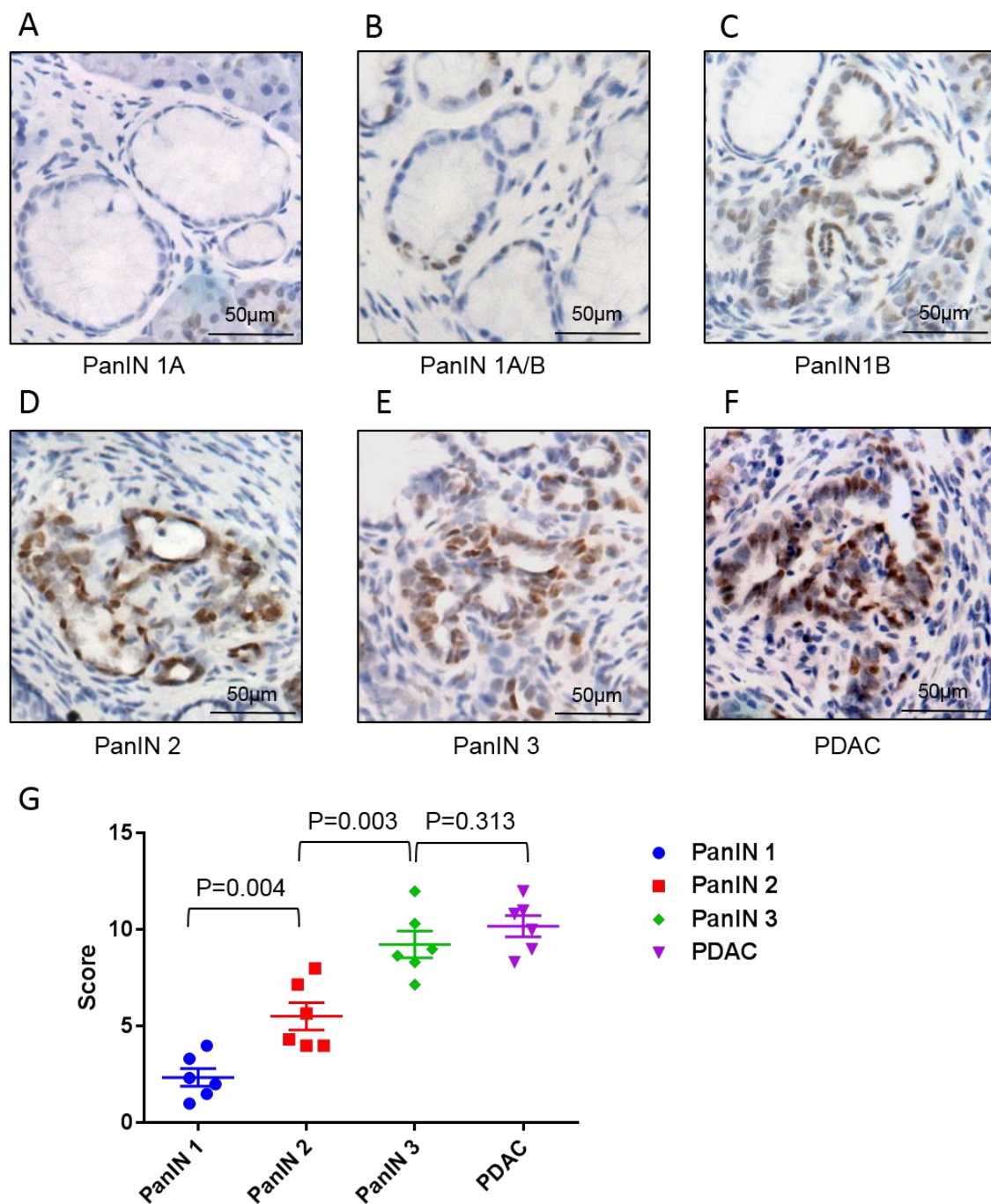


**Figure 6. PRMT1 is overexpressed in mouse PanIN and PDAC tissues.**

(A) Representative images of PRMT1 expression in a KC mouse pancreas tissue section.

(B) Representative images of PRMT1 expression in a KPC mouse pancreas tissue section. Red arrow indicates a PanIN lesion while the black arrow head indicates a PDAC lesion.

(C) Statistics of the average staining scores for different groups. N=6



**Figure 7. PRMT1 expression is gradually elevated during PanIN to PDAC progression in mouse pancreatic cancer models.**

Representative images of PRMT1 expression in different pancreas lesions from KC mice (A-D) and KPC mice (E and F).

(G) Statistics of the average staining scores for different groups. N=6

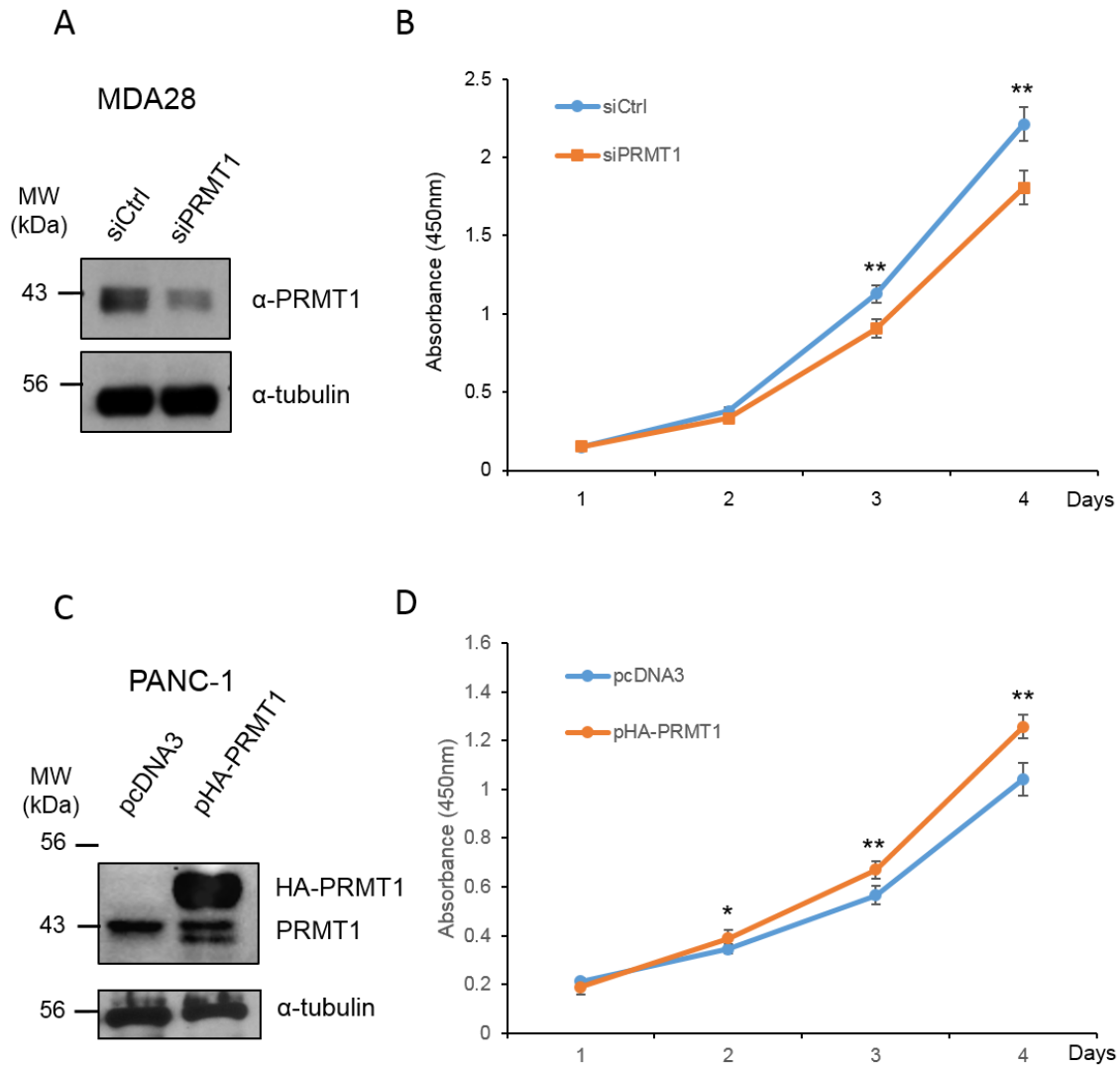
### **2.3.3 PRMT1 promotes cancer cell proliferation, migration and invasion *in vitro*.**

Above evidence clearly demonstrate that expression of PRMT1 increases with severity of dysplasia. During the progression of PanIN lesions, the proliferation, mobility and invasive ability of pre-cancerous cells gradually increase. The PanIN 3 lesion is used to be called Carcinoma *in situ* or intraductal carcinoma, which reflect the cancerous identity of these cells, but the basement membrane surrounding the lesions has not been broken through at this stage. When the integrity of basement membrane is destroyed, and cancer cells protrude to the surrounding tissues, it can be defined as invasive tumor, and tumor metastasis begins from this time point. Based on the observation that PRMT1 is gradually overexpressed during PanIN progression, I speculate that PRMT1 may have a promoting role in tumor proliferation, migration and invasion.

To test this hypothesis, MDA28 cells, a cell line with relatively high PRMT1 expression, were transfected with siRNA targeting PRMT1 (siPRMT1) or control siRNA (siCtrl), and PANC-1 cells, a cell line with relatively low PRMT1 expression, were transfected with PRMT1 expression vector (pHA-PRMT1) or control vector (pcDNA3), respectively (Fig.8A and C). Cell proliferation was measured by CCK-8 method for four consecutive days after transfection (Fig.8B and D). Results showed that overexpression of PRMT1 could increase cancer cell proliferation *in vitro*, while knockdown of PRMT1 did the opposite.

Meanwhile, the transfected cells were wounded by scratching and maintained for 12 hours. Cell migration ability was assessed by measuring the cell-free areas in at least three fields under microscopy. The results showed that knockdown of PRMT1 attenuated the flattening and migration of MDA28 cells (Fig.9A), whereas ectopic expression of PRMT1 strongly promoted the flattening and migration of PANC-1 cells (Fig. 10A). Similar results were observed in Boyden chamber invasion assay. The invasive ability of siPRMT1-transfected MDA28 cells were significantly attenuated (Fig.9B), whereas the

level of invasion in PRMT1-transfected PANC-1 cells were much higher than those of control cells (Fig. 10B).



**Figure 8. Influence of PRMT1 expression on PDAC cell proliferation *in vitro*.**

(A) MDA28 cells were transfected with siCtrl or siPRMT1, and knockdown of PRMT1 was conformed by Western blot.

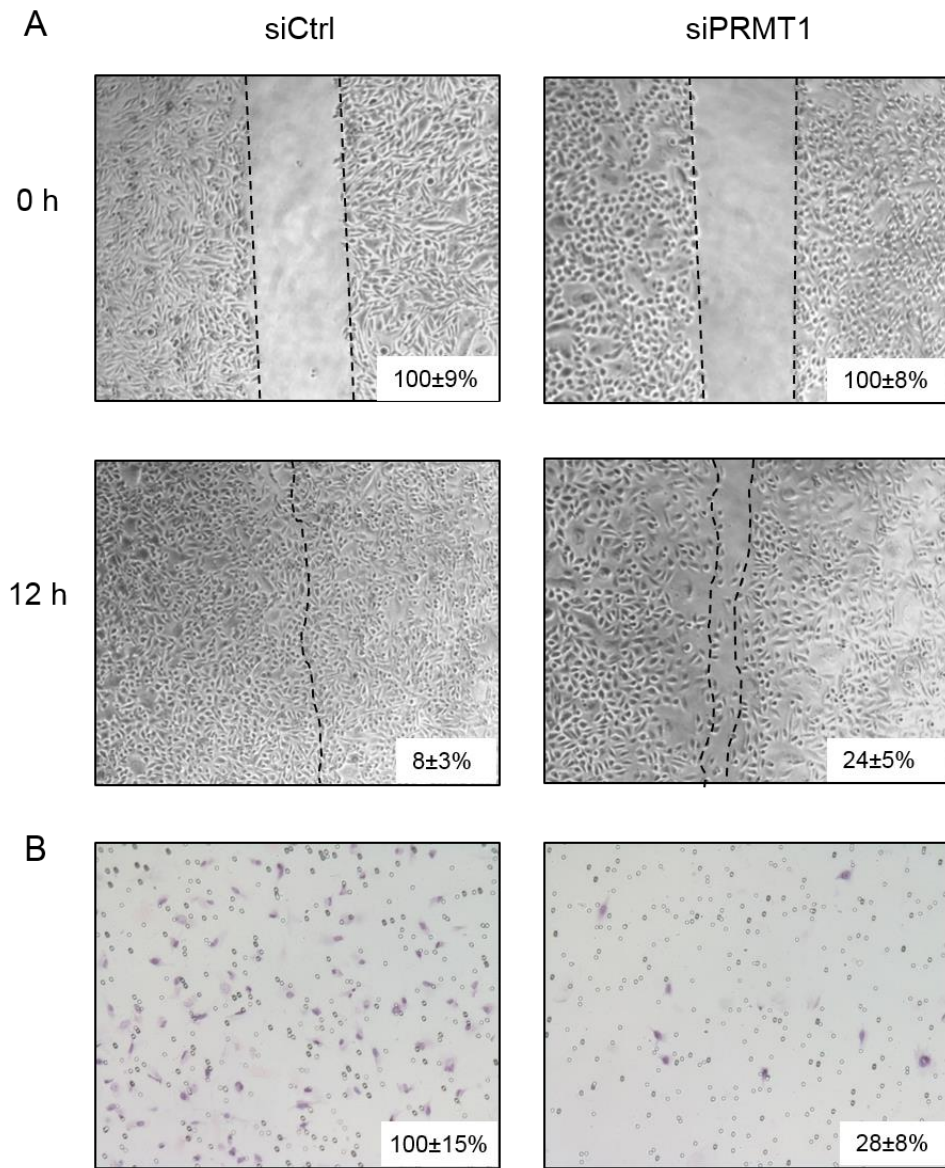
(B) *In vitro* cell proliferation of MDA 28 cells was measured by CCK-8 for four days.

(C) PANC-1 cells were transfected with pcDNA3 or pHA-PRMT1, and overexpression of PRMT1 was conformed by Western blot.

(D) *In vitro* cell proliferation of PANC-1 cells was measured by CCK-8 for four days.

\*  $P < 0.05$ , \*\*  $P < 0.01$



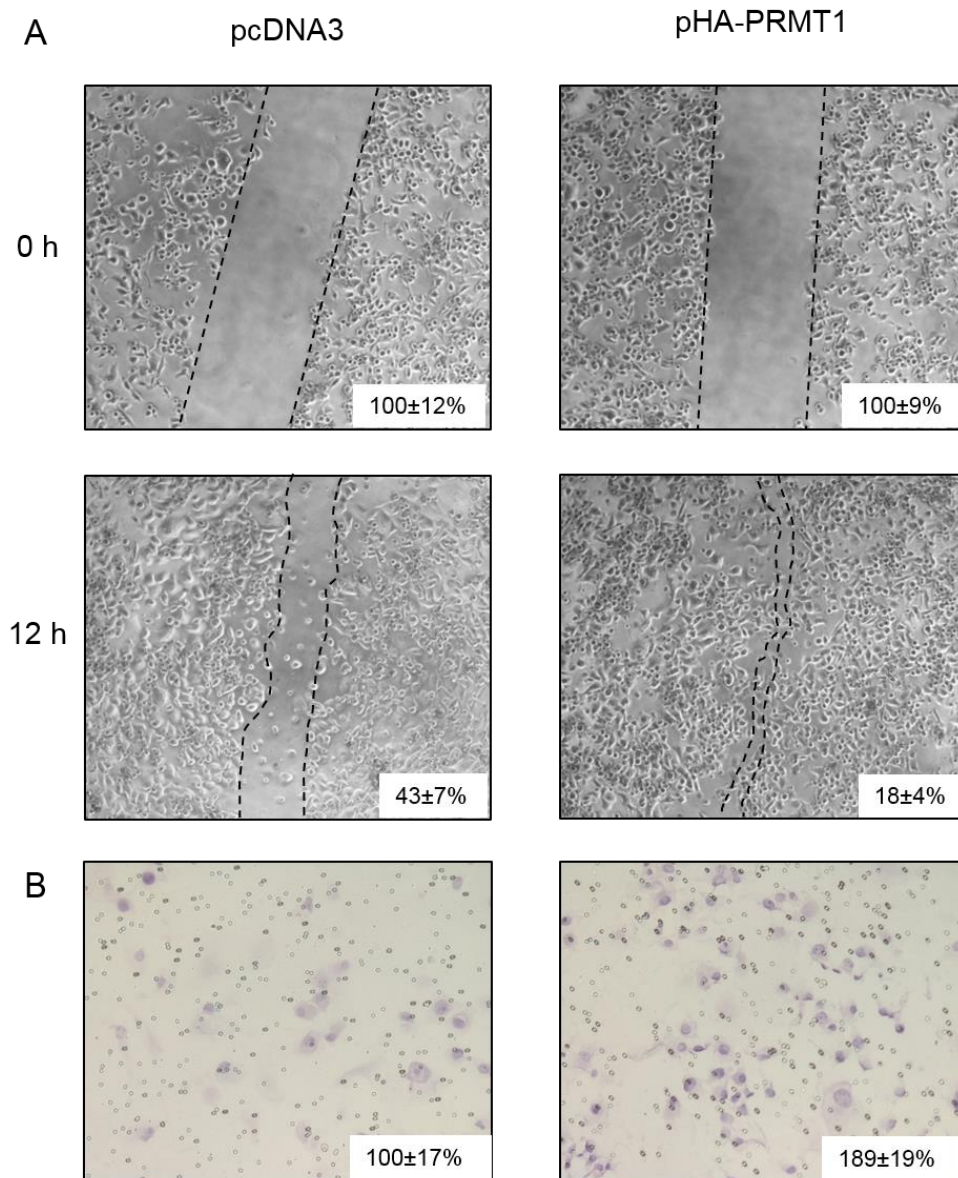


**Figure 9. PRMT1 knockdown decreases MDA28 cell migration and invasion *in vitro*.**

MDA28 cells were transfected with siCtrl or siPRMT1.

**(A)** Wound areas were photographed at 0 h and 12 h. Inserted numbers represented percentage of mean gap area  $\pm$  SD of triplicates.

**(B)** Boyden chamber invasion assays were performed, and photos of invaded cells stained by H&E were shown. Inserted numbers represent relative invasion measured by calculating ratios of siPRMT1 cell numbers to siCtrl cell numbers as mean  $\pm$  SD of triplicates.



**Figure 10. PRMT1 overexpression increases PANC-1 cell migration and invasion *in vitro*.**

PANC-1 cells were transfected with siCtrl or siPRMT1.

**(A)** Wound areas were photographed at 0 h and 12 h. Inserted numbers represent percentage of mean gap area  $\pm$  SD of triplicates.

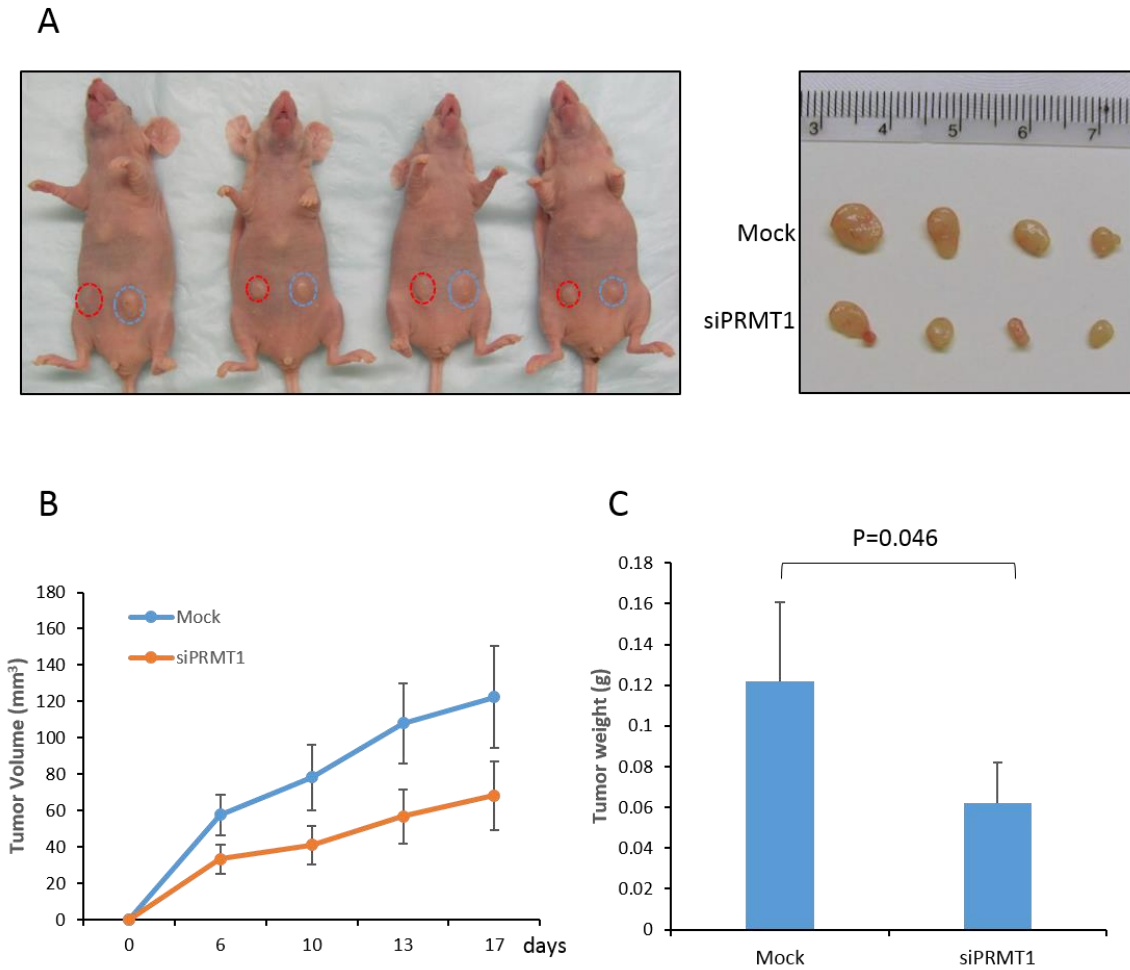
**(B)** Boyden chamber invasion assays were performed, and photos of invaded cells stained by H&E were shown. Inserted numbers represent relative invasion measured by calculating ratios of siPRMT1 cell numbers to siCtrl cell numbers as mean  $\pm$  SD of triplicates.



#### **2.3.4 Inhibiting PRMT1 expression or enzymatic activity decreases PDAC tumor growth *in vivo*.**

The *in vitro* data that knockdown of PRMT1 can inhibit tumor cell proliferation, migration and invasion prompt me to further study the outcome of targeting PRMT1 *in vivo*. MDA28 cells were transfected with either siCtrl or siPRMT1, and then injected subcutaneously into the flanks of nude mice. The tumor growth was monitored for 17 consecutive days. The results showed that knockdown of PRMT1 dramatically inhibited the growth of MDA28 cells *in vivo* (Fig.11).

Since dysregulation of PRMTs, including PRMT1, has been linked to many human diseases, such as cancer, the development of PRMT chemical modulators is a steady progress in the past several years. Although some pan-PRMT inhibitors showed cytotoxicity to various cell types, due to the high homology among PRMT family members, how to achieve high potency and selectivity to a specific PRMT protein is still a great challenge in drug development. Two small molecule compound, furamidine, also known as DB75, and TC-E5003 were recently identified to be PRMT1 specific inhibitors that have very low inhibition on other major PRMT members, like PRMT5 and CARM1/PRMT4 (68, 69). The anti-cancer activity of these two drugs were tested in PDAC models both *in vitro* and *in vivo*. After DB75 treatment for 48 hours, cultured cancer cells showed typical apoptosis phenotype (Fig. 12A). In addition, the cytotoxicity assay showed that both DB75 and TC-E5003 were toxic to several PDAC cell lines, with IC<sub>50</sub> value at micromole level (Fig.12B and C). Particularly, BxPC-3 cells, which have wild type KRAS protein, showed a relative insensitivity to these drugs. Whether this gene mutation is responsible for the insensitivity will be a subject for further investigation.



**Figure 11. Knockdown of PRMT1 by siRNA inhibits MDA28 subcutaneous tumor growth.**

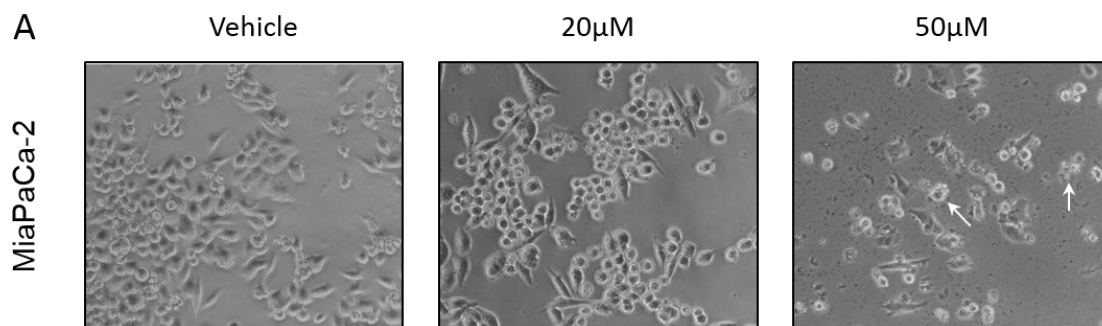
MDA28 cells were transfected with either siCtrl or siPRMT1, and then injected subcutaneously into nude mice to grow for 17 days.

**(A)** Tumors were photographed at day 17. Blue circles indicate the Mock tumor group and red circles indicate the siPRMT1 tumor group.

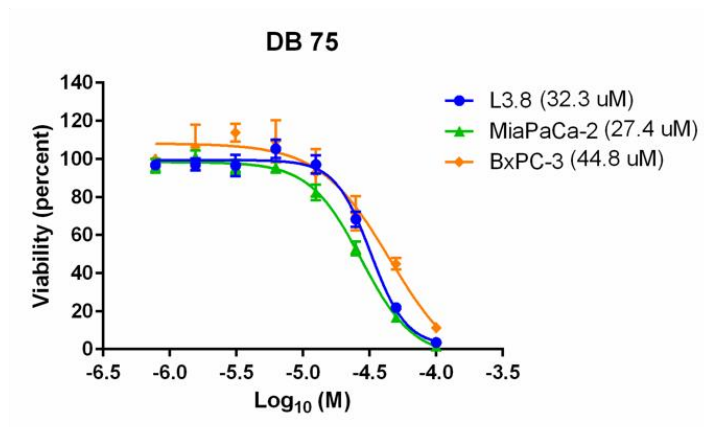
**(B)** Tumor volumes were monitored for 17 days after injection.

**(C)** Tumor weights were measured at day 17.

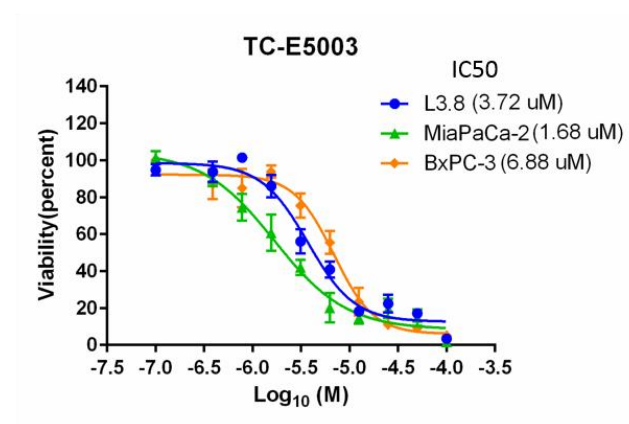
Since DB75 was previously used as an anti-parasite drugs in human, its biological safety should be acceptable. Therefore its anti-cancer effect was tested in a mouse model. Mouse metastatic pancreatic cancer H7 cells were injected subcutaneously into C57BL/6 mouse, and low-dose (5mg/kg) and high-dose (20mg/kg) DB75 were intraperitoneally administrated to the mice twice every week for three week. During this period, tumor sizes were monitored and tumor weights were measured at the end time point. Results showed that DB75 did not affect the expression level of PRMT1 (Fig. 13A), which is complied with previous report that this drug mainly targets PRMT1 enzymatic activity, not its expression (69). However, administration of DB75 dose-dependently inhibited H7 tumor growth *in vivo*, and showed no obvious side effects during this time window (Fig.13B-D).



**B**



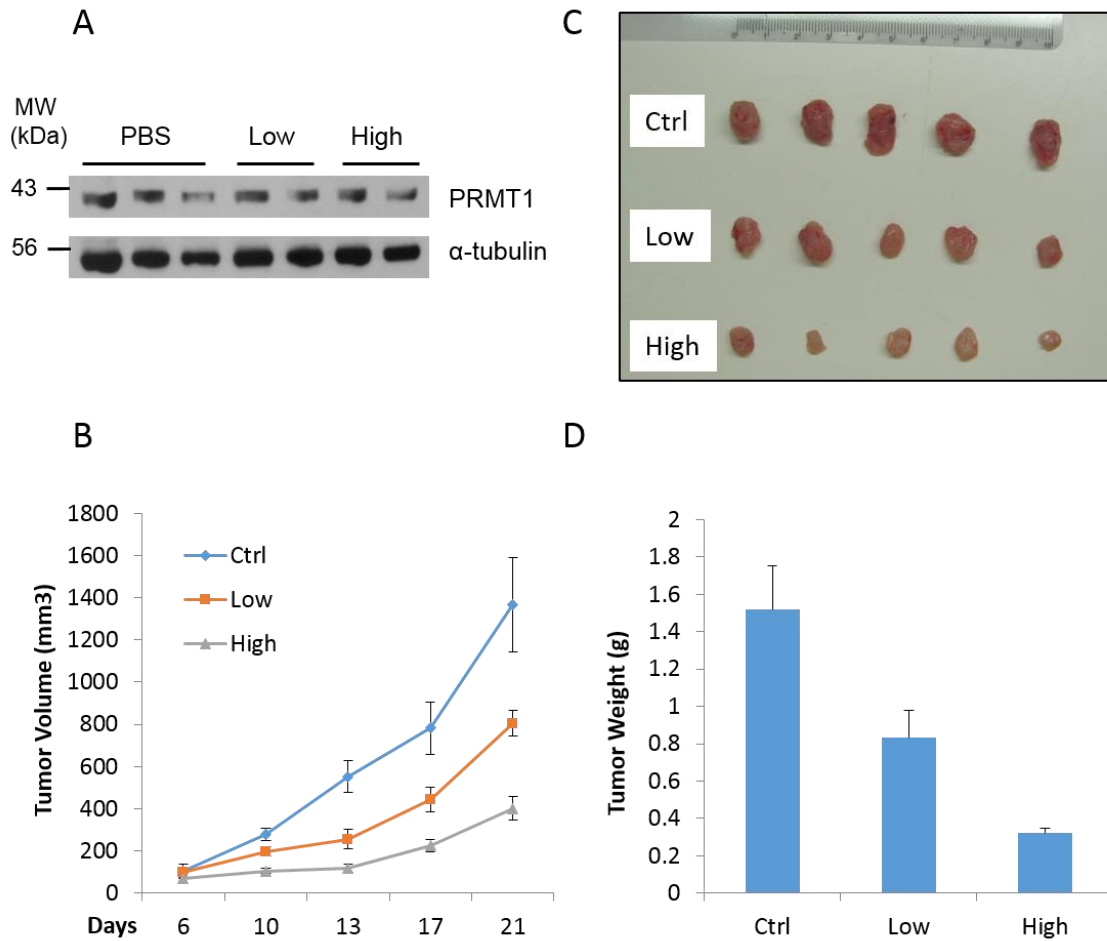
**C**



**Figure 12. PRMT1 specific inhibitors show cytotoxicity against PDAC cells *in vitro*.**

(A) DB75 is cytotoxic to MiaPaCa-2 cells, and it induces cell apoptosis (white arrows) at high concentration.

(B and C) IC50 values of DB75 and TC-E5003 on three PDAC cell lines were determined.



**Figure 13. PRMT1 inhibitor, DB75, decreases H7 subcutaneous tumor growth.**

Mouse pancreatic cancer H7 cells were injected subcutaneously into C57BL/6 mice to grow for three weeks. Vehicle or low-dose (5mg/kg) and high-dose (20mg/kg) DB75 was i.p. administrated during this period.

(A) Expression of PRMT1 in tumors from different groups was measured by Western blot.

(B) Tumor volumes were monitored for three weeks.

(C) Tumors from different groups were photographed at day 21.

(D) Tumor weights of different groups were measured at day 21.

## 2.4 DISCUSSION

PRMT1 is the primary type I arginine methyltransferases in mammals, and it has a broad spectrum of protein substrates that can be methylated at certain arginine residues. Overexpression of PRMT1, or certain splicing variants of it, has been reported in many cancer types, such as breast, prostate, lung, colon, bladder cancer and leukemia (30). However, the expression and function of PRMT1 in pancreatic cancer have not been demonstrated.

In this chapter, human tissue samples from several types of gastrointestinal (GI) cancers, primarily pancreatic cancer, and multiple human PDAC cell lines were utilized to show the overexpression of PRMT1 in PDAC. Moreover, statistical analysis shows that high expression of PRMT1 is correlated with shorter overall survival time and poorer differentiation grade of PDAC patients. The IHC staining results from mouse pancreatic cancer models clearly demonstrate the gradually elevated levels of PRMT1 during PanIN progression. All these evidence indicate a pro-tumorigenic role of PRMT1 in PDAC development. Next, gain- and loss-of-function assays were performed to demonstrate the role of PRMT1 in promoting cancer cell growth, migration and invasion *in vitro*. Last, two recently identified small molecular PRMT1 specific inhibitors were tested on PDAC cells both *in vitro* and *in vivo*, and they showed cytotoxicity at micromolar level to PDAC cells.

The PRMT1 expression status and functions in GI cancers are less studied based on previous publications, and some controversy exists among these studies (70-73). For example, *PRMT1* gene expression was found to be upregulated in colon cancer tissues, and this upregulation was associated with poor prognosis (72, 73). Similarly, in human hepatocellular carcinoma (HCC) cells, overexpression of PRMT1 promoted cancer cell migration and invasion (71). However, another study in gastric cancer revealed a nuclear expression of PRMT1 in cancer cells, and those authors claimed that lower expression of

PRMT1 was associated with recurrence after adjuvant chemotherapy and poor prognosis (70). In my study, the IHC staining results clearly showed the overexpression of PRMT1 in pancreatic cancer tissues than normal acinar or ductal cells. The functional assay also demonstrated a tumor-promoting function of PRMT1 under my experimental conditions. From the data in my present study and previous reports, it seems that PRMT1 may have a dual function as a tumor promoter or suppressor. These phenomena may reflect a context- or cell type-dependent function of PRMT1, or distinct splicing variants of PRMT1 may be dysregulated in different cancer types so that the overall PRMT1 expression could not be used as a prognostic marker alone. All these possibilities warrant further study.

## **CHAPTER 3. HSP70 IS A NOVEL SUBSTRATE OF PRMT1**

### **3.1 INTRODUCTION**

The results in Chapter 2 outlined a tumor-promoting function of PRMT1 in pancreatic cancer. However, how this function is achieved at the molecular level is not known. Since PRMT1 is an enzyme, which is responsible for methylating certain arginine residues in its substrate proteins, it is logical to postulate that PRMT1's function in cancer development is mainly mediated by its enzymatic activity. The best known substrate for PRMT1 is histone H4. PRMT1 can specifically deposits a MMA or ADMA mark on histone H4 at arginine 3 (H4R3), which is a transcription activation marker (74). Although the underlying mechanisms that how H4R3 methylation activates corresponding gene transcription have not been completely elucidated, the protein complex containing methylarginine effector molecule TDRD3 and topoisomerase IIIB (TOP3B) is proved to play a role in this process (75, 76).

Large numbers of non-histone substrates of PRMT1 have also been identified. By methylating diverse proteins localized at different subcellular compartments, PRMT1 is involved in many biological processes, such as DNA damage repair, RNA processing, receptor signal transduction etc., which may contribute to tumor cell survival, migration and invasion (30). Compared with histone arginine methylation, which may affect gene expression at the transcription level, arginine methylation of non-histone proteins at the post-transcription level is a quick, energy-efficient and perhaps a reversible way to precisely regulate the function of a specific protein to help cells adapt to the ever-changing microenvironment. Thus to identify and study novel substrate proteins of PRMT1 that may function in cancer biology is the primary focus of this chapter.

As it is mentioned in Chapter 1, HSP70 family has several members that serve critical survival functions in cells, particularly cancer cells. Moreover, some studies also revealed



that lysine or arginine methylation of HSP70 may affect its function as a molecular chaperon or transcription co-activator (61, 62, 77, 78). It is plausible that these modifications expand or regulate the chaperon client scope of HSP70 proteins and therefore affect their biological functions. In my study, I found that at least four members of HSP70 family are binding partners of PRMT1. This observation can be interpreted as either HSP70 proteins may facilitate PRMT1 folding, or HSP70 proteins may be a new group of substrates of PRMT1.

In this chapter, first the protein-protein interaction of four representative HSP70 family members with PRMT1 is studied by Co-immunoprecipitation (Co-IP) assay in mammalian cells and GST pull-down assay *in vitro*. Next, the essential domain for interaction is determined by using a series of truncated HSP70 protein variants, and the structure of PRMT1-HSP70 complex is studied by molecular modeling. Last, the potential methylation sites are determined by mass spectrometry and *in vitro* methylation assay with corresponding peptides and recombinant proteins.

## 3.2 MATERIAL AND METHODS

### Plasmids and Cloning Procedures

Full-length HSPA1A (HSP70), HSPA5 (GRP-78), HSPA8 (HSC70) and HSPA9 (GRP-75) plasmids were purchased from OriGene Technologies, and they are in the pCMV6-Entry vector backbone with C-terminal Myc-DDK tag. GFP-PRMT1 and GST-PRMT1 plasmids were gifts from Dr. Mark T. Bedford at UT MD Anderson Cancer Center. The HSP70 and PRMT1 gene truncations were PCR-amplified by using Herculanase II Fusion Enzyme (Agilent Technologies) and inserted back to the original vectors or GEX-KG vector for bacterial expression. Arginine-to-alanine mutant HSP70 vectors were generated by overlap extension PCR method. All other plasmids used in this chapter have been described in Chapter 2.

### siRNAs, Antibodies, Peptides and Compounds

Anti-Myc and anti-HA mouse monoclonal antibodies were purchased from OriGene Technologies. Anti-HSP70 antibody was from Enzo Life Sciences. Anti-HSC70, anti-GRP78, anti-GRP75 and anti-GST antibodies were from Santa Cruz Biotechnology. Anti-asymmetric dimethyl arginine antibody, ASYM24, was from EMD Millipore Corporation. All other antibodies and siRNAs have been described in Chapter 2. HSP70 wild type and mutant peptides were custom synthesized by ApexBio Technology as

R3	GGRRGGFGGRGGFGGRGGFG
3RA	GGAGGGFGGAGGGFGGAGGGFG
R416	GVMTALIKRNSTIPTKQTQ
R416A	GVMTALIKANSTIPTKQTQ
R447	VLIQVYEGERAMTKDNNLLG
R447A	VLIQVYEGEAMTKDNNLLG
R458	MTKDNNLLGRFELSGIPPAP
R458A	MTKDNNLLGAFELSGIPPAP

### **Immuoprecipitation and Immunoblotting assay**

For immunoprecipitation assay, cells were lysed in Pierce IP Lysis Buffer (Thermo Fisher Scientific) supplemented with protease inhibitor cocktail, sodium fluoride, sodium orthovanadate, and phenylmethylsulfonyl fluoride (PMSF). Cell lysates were incubated with corresponding antibody or antibody conjugated magnetic beads overnight at 4°C on a rotator. If antibody were used for IP, Protein A/G plus agarose (Santa Cruz) was then added and the reactions were further incubated at 4°C for 2 h. After washing with PBST for four times, 5min each, protein complexes were released from agarose by boiling for 10min in 2x SDS-PAGE loading buffer or by elution with tag peptides. Immunoblotting was performed to detect specific protein in the complex as it is described in Chapter 2.

### **Protein Purification from Bacterial Cells or HEK293T Cells**

GST-tagged proteins were expressed in BL21 (DE3) bacterial cells (NEB) and induced by 100μM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Thermo Fisher Scientific). Then bacterial cells were lysed by Pierce pull-down lysis buffer supplemented with protease inhibitor cocktail, and purified by using Pierce Glutathione Magnetic Agarose Beads (Thermo Fisher Scientific). Protein complexes were eluted by 50 mM reduced glutathione. Myc- or HA-tagged proteins were expressed in HEK293T cells by transient transfection and cells were lysed in lysis buffer and purified by Myc or HA peptide conjugated magnetic beads and washed extensively with PBST. Purified proteins were eluted by Myc or HA peptide (ApexBio Technology).

### **Immunofluorescence assay**

Cells grown in Falco 4-well chambered cell culture slides were fixed with 4% paraformaldehyde in PBS for 10min and rendered permeable by further treatment with 0.2% Triton X-100 for 10min. Then fixed cells were blocked by incubation with 5% BSA

solution for 30 min. The PRMT1 antibody and HSP70 antibody (Enzo Life Sciences, ADI-SPA-810-F) were 1:200 diluted and mixed in 5% BSA solution and incubated with cells overnight at 4°C. After washing with PBST for three times, cells were incubated with Alexa 594-labelled anti-rabbit IgG and Alexa 488-labelled anti-mouse IgG antibodies (Thermo Fisher Scientific) for 2h at room temperature with gentle shaking. Cells were then washed, mounted with prolong gold antifade reagent with DAPI (Thermo Fisher Scientific), viewed, and photographed under a fluorescence microscope (Leica).

### ***In vitro* methylation assay**

Synthesized peptides or purified recombinant proteins were incubated with 2µg PRMT1 recombinant protein (Origene Technologies) and 2µCi S-[methyl-<sup>3</sup>H]-adenosyl-methionine (Perkin Elmer) in 20µl methylation buffer (50mM Tris·HCl, pH 8.0, 150mM NaCl, 1mM EDTA). Reactions were incubated at 30°C for 3h. Results were measured by either liquid scintillation counting (LSC) or autoradiography. For LSC, 10µL mixture was dotted onto a small circular nitrocellulose membrane and washed three times with methylation buffer. Then the membrane was air dried and immersed in ScintiVerse II Cocktail (Thermo Fisher Scientific) in a glass scintillation vial and measured by Liquid Scintillation Counter (Beckman Coulter). The DPM for each peptide is calculated as net DPM = DPM (wild type) – DPM (mutant). For autoradiography, the reaction mixture was boiled in loading buffer for 5min and then separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Membrane was treated with EN3HANCE Spray (Perkin Elmer) and exposed to film at -80°C for 7-10 days.

### **Protein sequence alignment**

All the protein sequences were retrieved from UniProt Protein Database in FASTA format. Multiple sequence alignment was performed use online web tool Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).

### **Mass Spectrometry Analysis**

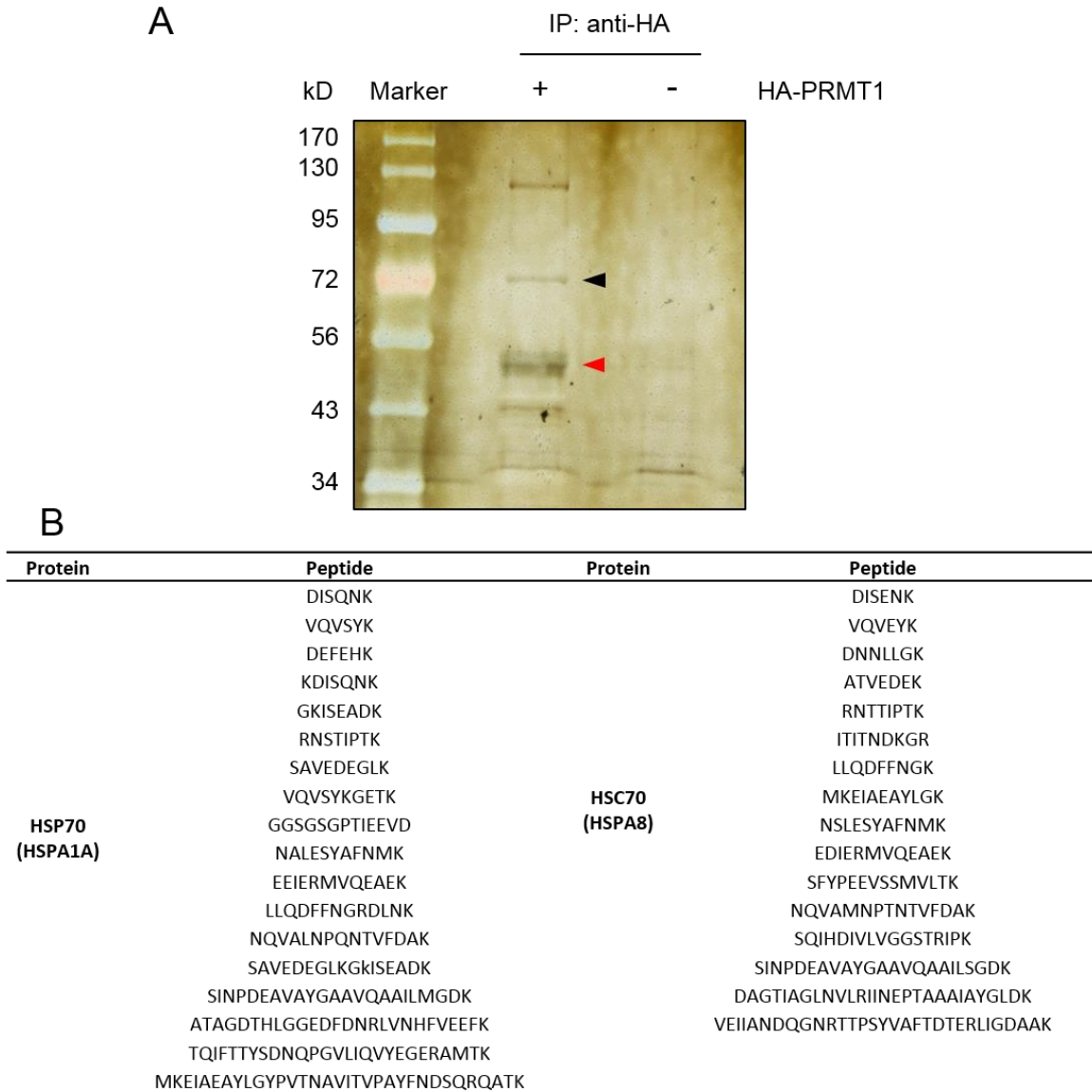
HEK293T cells were co-transfected with HA-PRMT1 and Myc-HSP70 plasmids and cultured for 48h. Then cells were lysed in Pierce IP Lysis Buffer supplemented with protease inhibitor cocktail. Cell lysate was incubated with anti-Myc antibody conjugated magnetic beads overnight at 4 °C, followed by washing five times with lysis buffer. Immunoprecipitates were boiled in SDS sample buffer for 5min and resolved by 10% SDS-PAGE gel, followed by Coomassie blue staining. Band corresponding to HSP70 was excised and subjected to in-gel digestion with trypsin. Then samples were sent to the University of Texas MD Anderson Cancer Center Proteomics and Metabolomics Facility for MALDI-TOF analysis using an Ultimate capillary LC system (LC Packings) coupled to a QSTARXL quadrupole time-of-flight mass spectrometer (Applied Biosystem/MDS Sciex).

### **3.3 RESULTS**

#### **3.3.1 PRMT1 interacts with four members of HSP70 protein family.**

Histones are classic methylation substrates of PRMT1. To identify novel non-histone substrates of PRMT1, first I screened for PRMT1-interacting proteins using affinity purification combined with mass spectrometry. I found that PRMT1 bound to endogenous HSP70 and HSC70 in HEK293T cells (Fig.14). My observation is consistent with previous studies that HSP70 protein was identified to interact with PRMT1 in other systems (79-81). Moreover, some studies also identified other HSP70 family member, such as GRP75 and GRP78, in protein complexes with PRMT1 (79-81). To further validate these observations, I tested the protein-protein interactions (PPIs) between four major HSP70 family members (HSP70, HSC70, GRP75 and GRP78) and PRMT1 using co-immunoprecipitation (Co-IP) assay.

First, Myc-tagged HSP70, HSC70, GRP75 and GRP78 were co-transfected to human 293T cells with HA-tagged PRMT1, respectively. Co-IP results showed that these four Myc-tagged exogenous proteins could bind to exogenous HA-PRMT1 (Fig.15A). Next, HA-tagged PRMT1 was transfected to 293T cells, and binding of HA-PRMT1 with four endogenous HSP70 family members was demonstrated (Fig.15B). Finally, the endogenous interaction of HSP70 and PRMT1 under normal culture conditions was confirmed in pancreatic cancer cell line L 3.7 (Fig. 15C). These evidence demonstrate that at least four members in HSP70 protein family can interact with PRMT1 in human cells. Since HSP70 is the representative and best studied protein in this family, and its dysregulation is observed in pancreatic cancer, my following study will mainly focus on HSP70 and the possible PTMs on it.

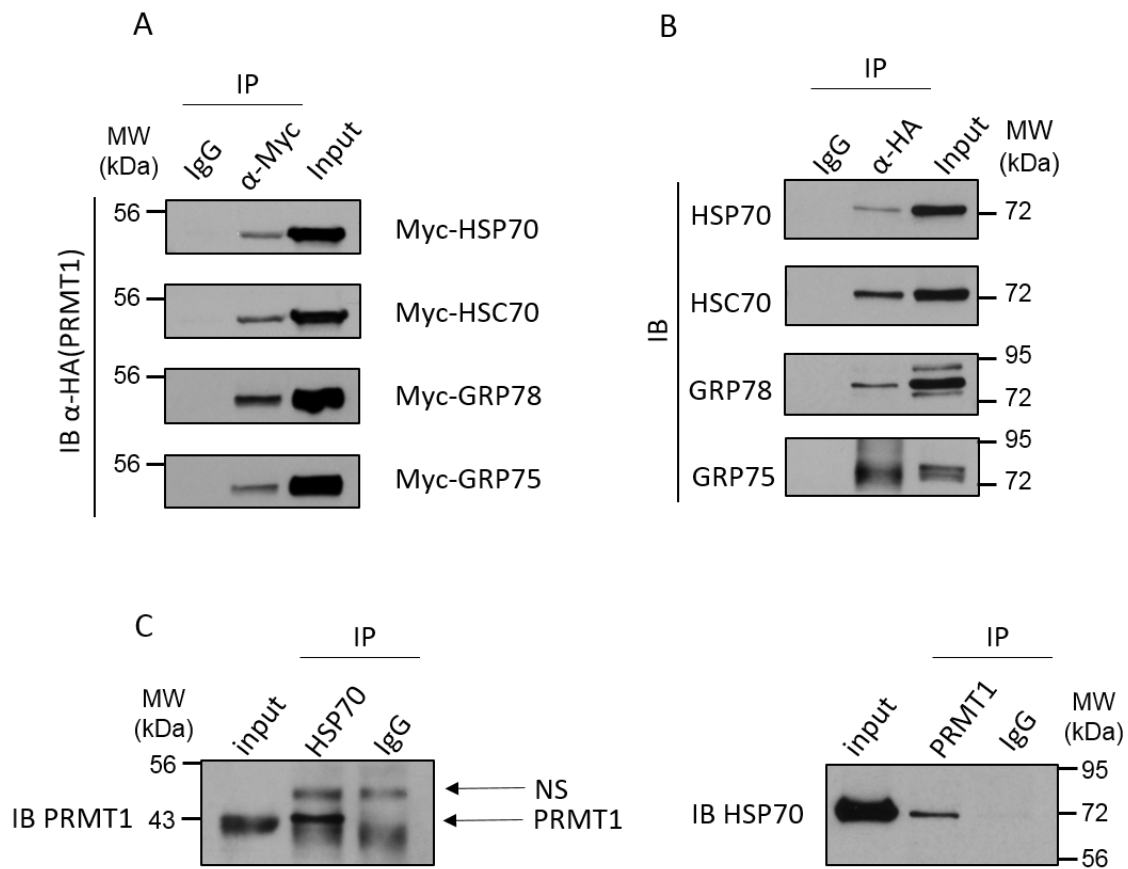


**Figure 14. PRMT1 binds to HSP70 and HSC70.**

HA-PRMT1 and HA empty vectors were transfected into 293T cells, respectively. Cell lysates were immunoprecipitated with anti-HA magnetic beads, washed and eluted by HA peptide.

(A) Silver staining of the eluted proteins from two groups. The band indicated by a black arrowhead was identified to be HSP70 and HSC70 by mass spectrometry. HA-PRMT1 is indicated by a red arrowhead.

(B) HSP70 and HSC70 peptide sequences identified by mass spectrometry.



**Figure 15. PRMT1 interacts with four HSP70 protein family members.**

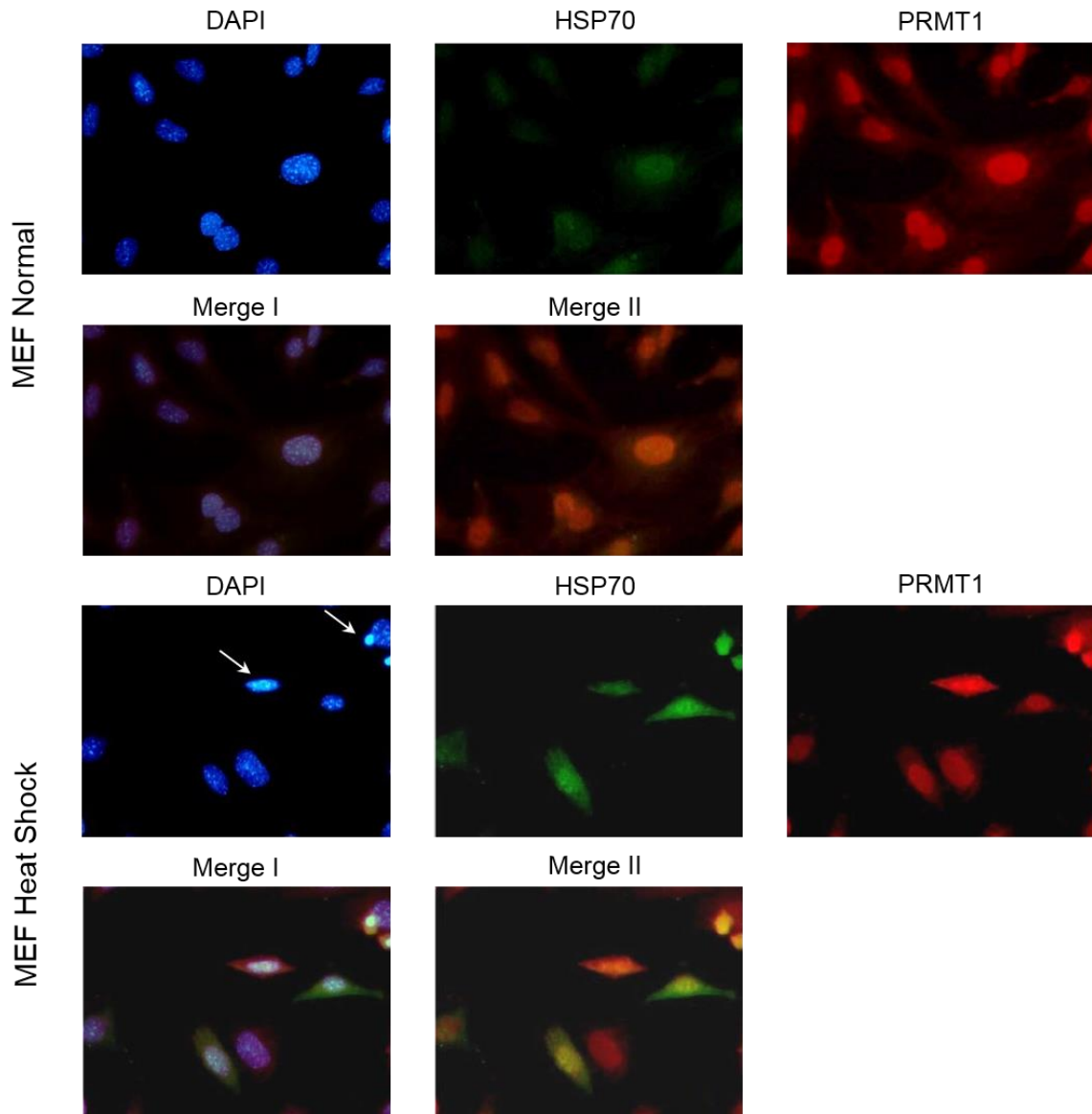
(A) Validation of PRMT1-HSP70s interaction by Co-IP assay. HA-tagged PRMT1 and Myc-tagged HSP70s were co-expressed in 293T cells, and cell lysates were immunoprecipitated by anti-Myc antibody and immunoblotted by anti-HA antibody.

(B) Ectopically expressed HA-PRMT1 protein can bind to endogenous HSP70s .

(C) Endogenous interaction between PRMT1 and HSP70 was observed in L3.7 cells measured by immunoprecipitation. NS, non specific band

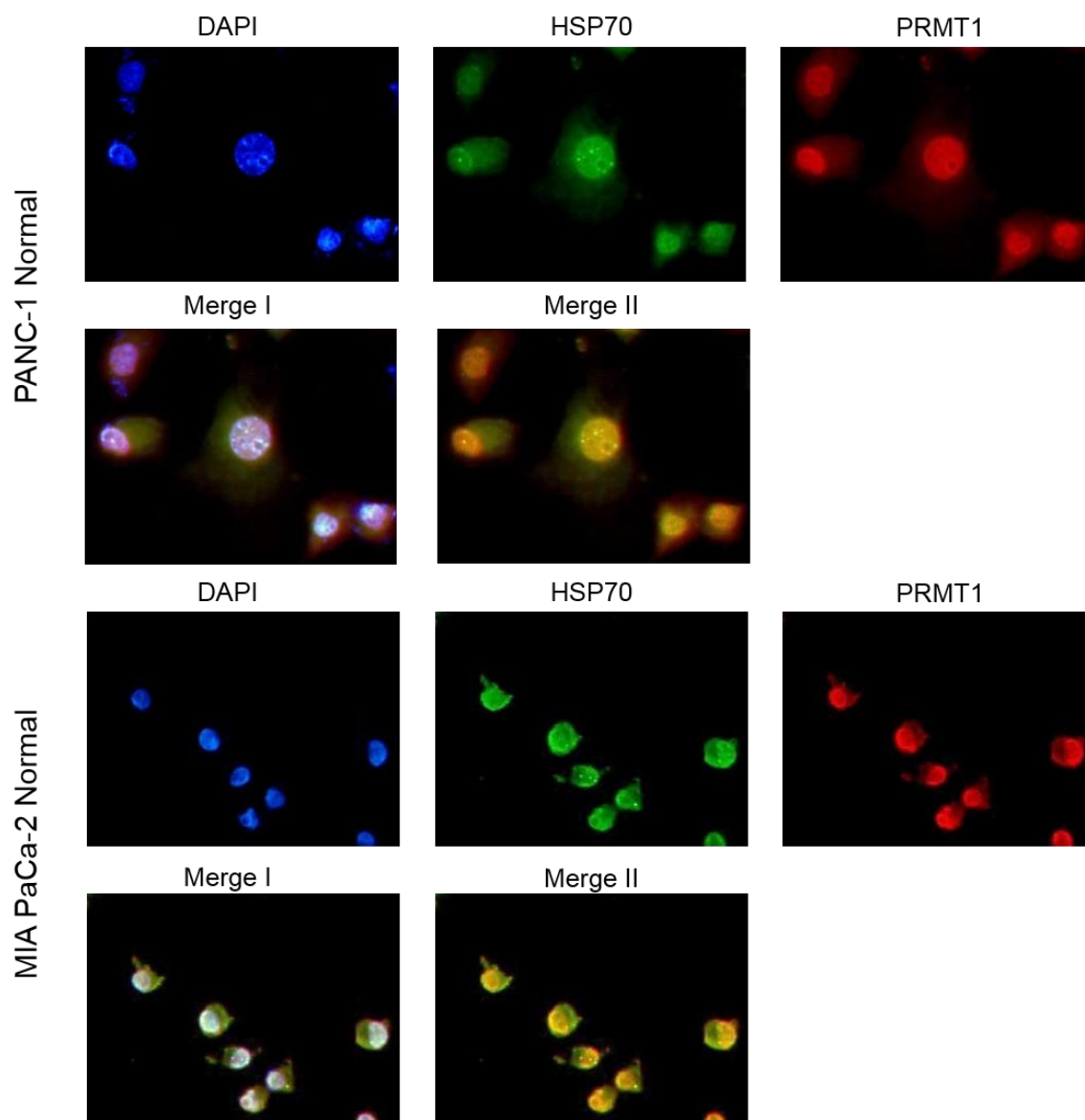


In addition to Co-IP assay, the subcellular localization of PRMT1 and HSP70 was also studied in both normal cells and pancreatic cancer cells. It showed that in mouse embryonic fibroblast (MEF) cells, HSP70 expression was very low under normal culture condition, and was dramatically induced after heat shock treatment (Fig.16). The HSP70 protein was distributed in both cytoplasm and cell nucleus, but was more nuclear accumulated after heat shock treatment, which is consistent with previous reports (82, 83). In contrast, HSP70 protein was mainly localized in cell nucleus in PANC-1 and MIA PaCa-2 pancreatic cancer cells, and its expression was constantly high even without heat shock treatment (Fig.17). In all mouse and human cells, PRMT1 was mainly expressed in cell nucleus, while it was also observed in the cytoplasm and/or cell membrane at a lower level (Fig. 16 and 17). Moreover, the expression level of PRMT1 was not affected by heat shock treatment when compared with HSP70. The Immunofluorescence results not only indicate the possible interaction between HSP70 and PRMT1 due to their co-localization, but also imply important role of HSP70 in cell nucleus of cancer cells.



**Figure 16. Immunofluorescence microscopy of HSP70 and PRMT1 in MEF cells.**

The endogenous HSP70 (green) and PRMT1 (red) were detected by immunofluorescence microscopy using HSP70 and PRMT1 specific antibodies in MEF cells under normal culture condition or after heat shock treatment (1h at 43°C and then recover 6h at 37°C). White arrows indicate representative cells undergoing apoptosis after heat shock, which are featured by cell shrinkage and condensed nuclei. Merge I is to merge three fluorescence while Merge II is to merge green and red fluorescence.



**Figure 17. Immunofluorescence microscopy of HSP70 and PRMT1 in PANC-1 and MIA PaCa-2 cells.**

The endogenous HSP70 (green) and PRMT1 (red) were detected by immunofluorescence microscopy using HSP70 and PRMT1 specific antibodies in PANC-1 (upper panel) and MIA PaCa-2 (lower panel) cells under normal culture condition. Merge I is to merge three fluorescence while Merge II is to merge green and red fluorescence.

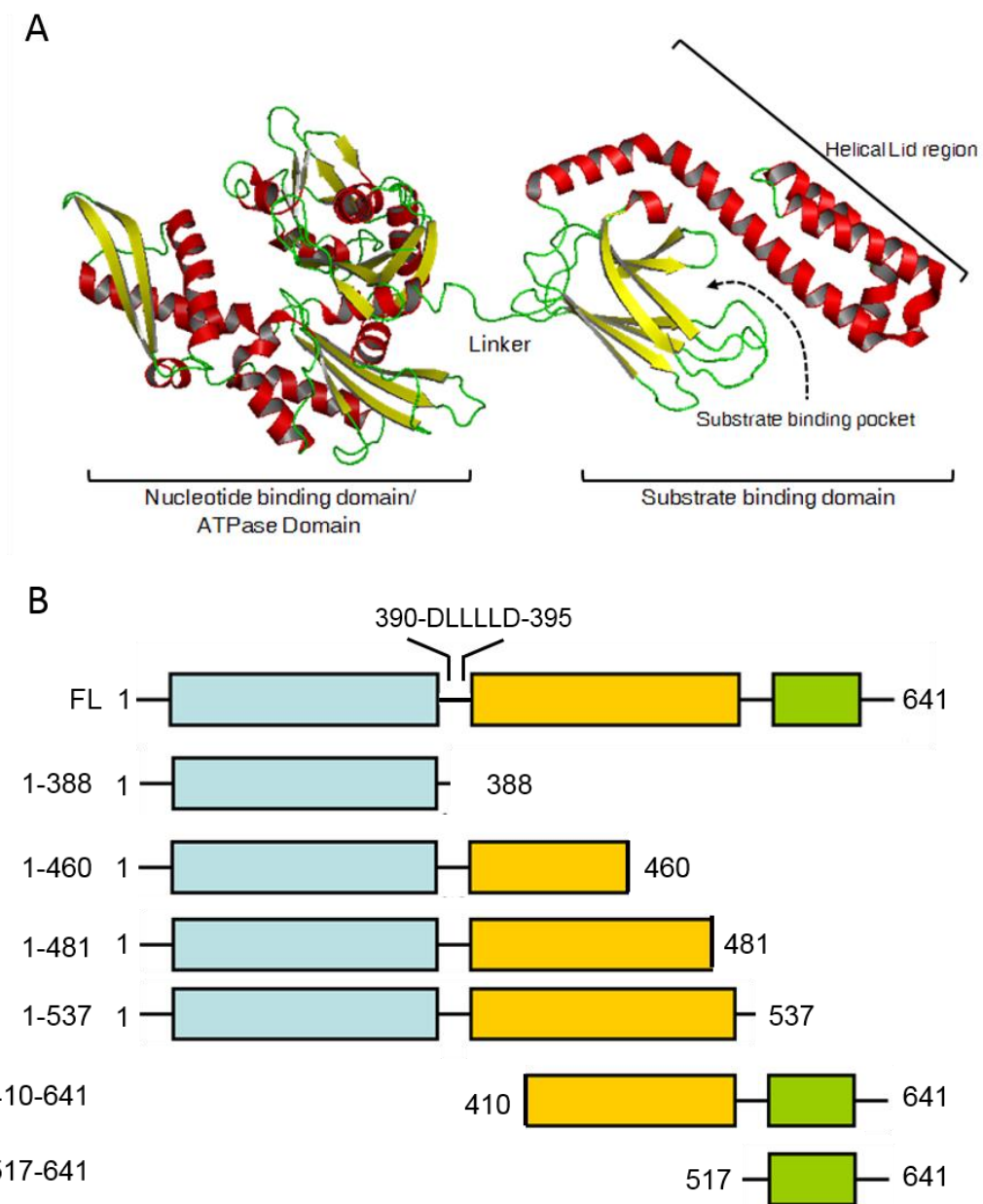
### **3.3.2 Binding domain of HSP70 with PRMT1 is determined.**

HSP70 mainly functions as a molecular chaperon to facilitate folding and re-folding of other proteins. There is a nucleotide binding domain (NBD) at the amino terminus of HSP70 that has an ATPase function, and a substrate binding domain (SBD) at the carboxyl terminus that is responsible for binding of client protein or peptides, and a linker region between these two domains (Fig. 18A)(84). The chaperon function of HSP70 is an energy-consuming process that depends on the hydrolysis of ATP. Thus modifications added to either the NBD or SBD of HSP70 may affect its affinity to ATP/ADP or substrate protein, respectively, and therefore influence its biological functions.

To identify the region of HSP70 that is required for its interaction with PRMT1, I generated a series of Myc-tagged deletion mutants of HSP70 and co-transfected them with HA-tagged PRMT1 to 293T cells (Fig. 18B). Immune complexes of HA-tagged PRMT1 were tested for the presence of Myc-tagged HSP70 fragments. As shown in Fig. 19A, the interaction assays suggested that amino acids M410-E460 of HSP70 are essential to mediate HSP70 interaction with PRMT1. Interestingly, I also found that deletion either the N-terminal part or C-terminal part regarding motif M410-E460 dramatically increased its binding affinity to PRMT1 when compared with full-length HSP70 (compare lane 2, 4 with lane 7 in Fig. 19A). The reason may be that removal of the obstructive motif at either side can expose the binding domain more efficiently. Thus it is more accessible for PRMT1 to interact with the binding pocket. It is particularly reasonable when we consider the fact that the carboxyl terminus (~500-641 amino acids) composes a helical lid structure that may mask the binding domain and impede the possible protein-protein interaction.

To further pinpoint the binding motif for this interaction, amino acids 410 to 460 of HSP70 were divided into three fragments and similar interaction assays were performed. To my surprise, it seemed that all the fifty amino acids were contributive to the observed

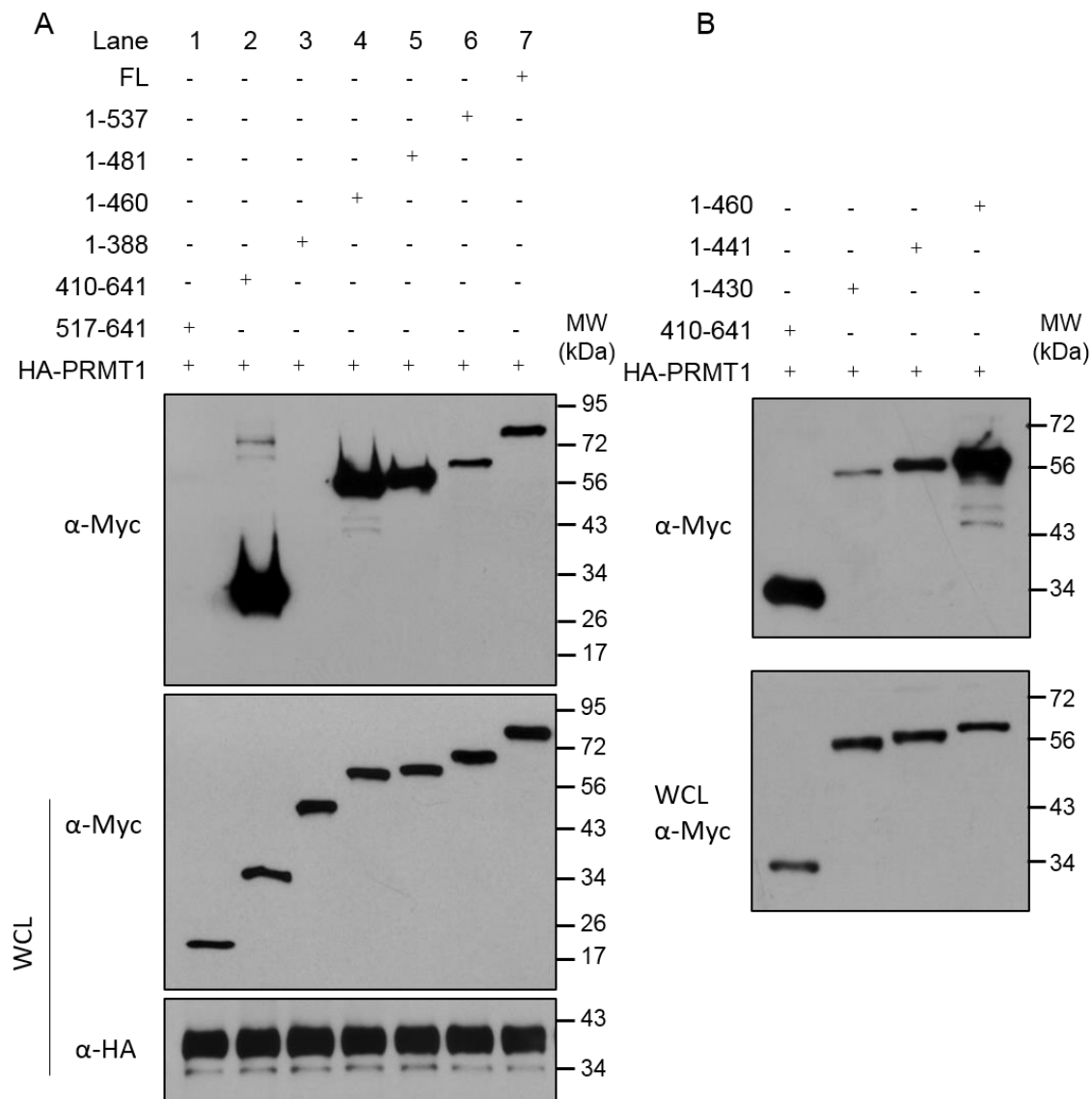
interaction, because binding affinity gradually increased when the carboxyl terminus extended from 430 to 441 and 460 (Fig. 19B). I speculate that there may be a small core binding motif in domain 410-460, and the other proximal amino acids around it are also supportive to this interaction.



**Figure 18. Structure and deletion mutants of HSP70 protein.**

(A) Structure of HSP70 shows the N-terminal NBD domain, C-terminal SBD domain and linker region between them. Structure obtained from HSPiR database (84).

(B) A series of deletion mutants of HSP70 used in interaction assays. Blue boxes indicate the NBD domain. Yellow boxes indicate substrate binding region and green boxes indicate the helical lid region. FL, full length



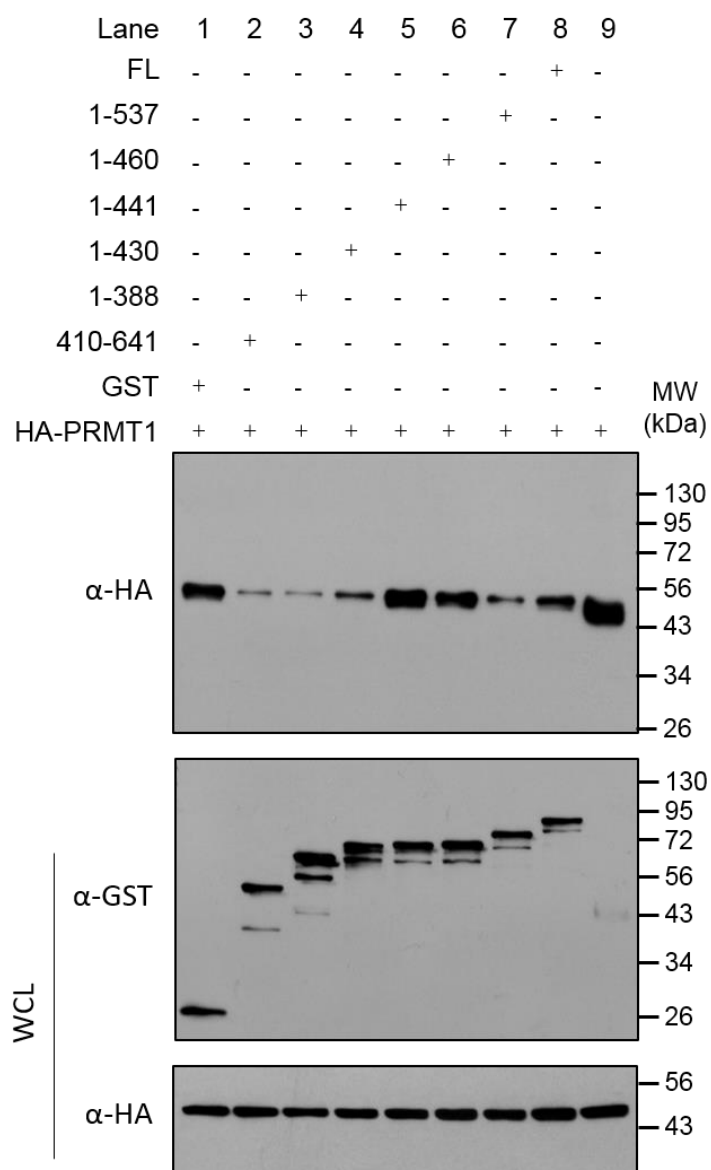
**Figure 19. Amino acids 410-460 is required for HSP70-PRMT1 interaction.**

(A) Mapping the protein domain of HSP70 that binds to PRMT1. Myc-tagged full-length (FL) or deletion mutants of HSP70 and HA-tagged PRMT1 were co-transfected to 293T cells, and cell lysates were immunoprecipitated with HA antibody and then blotted with anti-Myc antibody. WCL, whole cell lysate.

(B) Similar interaction assay was performed with two more HSP70 deletion mutants ranging between amino acids 410-460.

To further confirm the mapping results I got in 293T cells and determine whether the binding of HSP70 with PRMT1 is a direct interaction, *in vitro* GST pull-down assay was performed. A series of HSP70 deletion mutants were expressed in bacteria as GST fusion proteins. Equal amount of fusion proteins of each deletion mutant were immobilized to glutathione beads, and then incubated with cell lysate from 293T cells transfected with HA-tagged PRMT1 (Fig. 20). Immunoblotting results showed that the GST tag had a medium level interaction with HA-PRMT1 protein (Fig. 20 Lane1). This is not desired in the assay, but is consistent with a previous study showing that PRMT1 could interact with Glutathione S-transferase P (81). If we exclude the GST tag effect, the pull-down data recapitulated the mapping results in 293T cells, in which the 1-441 and 1-460 fragments had the best binding affinity with PRMT1, while 1-388 fragment had the lowest, maybe a background level, binding affinity. The only difference came from the 410-641 fragment. In mammalian cells, it had a very high affinity with PRMT1, but as a GST fusion protein, the binding affinity dramatically decreased (compare Fig. 20 Lane 2 with Fig. 19A Lane 2). The reason for this difference may be that fusion N-terminus of 410-641 fragment to GST tag, a large peptide roughly 26 kDa, can alter the local polypeptide conformation near 410-460 binding motif, and therefore influences its binding affinity with PRMT1. While in mammalian expression system, the HA tag is very small (less than 4 kDa) and is attached to the C-terminus of 410-641 fragment, which probably has little effect on its binding activity.





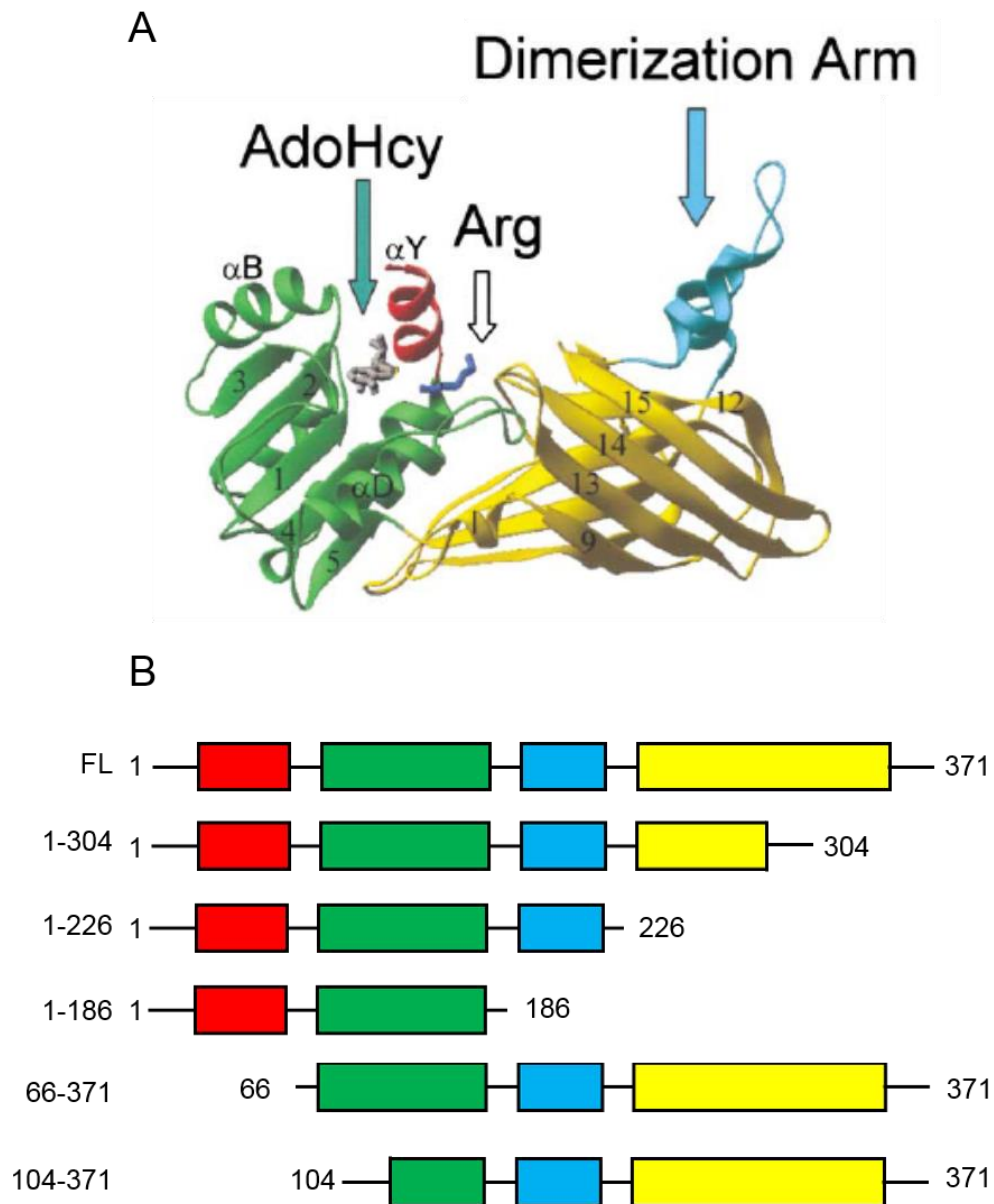
**Figure 20. GST pull-down to map the binding motif of HSP70 to PRMT1.**

Cell lysates from HEK293T cells transfected with HA-tagged PRMT1 were mixed with purified GST or GST-tagged HSP70 deletion mutants immobilized on glutathione beads. Samples were electrophoresed and immunoblotted with anti-HA antibody. Levels of input protein are shown by immunoblotting with anti-HA or anti-GST antibody.

With similar method, several deletion mutants of human PRMT1 were generated to identify the interaction motif of PRMT1 required for its binding to HSP70 (Fig. 21). The strategy to construct these deletion mutants vectors are based on the functional domain distribution of PRMT1. However, it was so surprising to find that some truncated PRMT1 proteins could not be stably expressed in 293T cells under my experimental condition (Fig. 22). Compared with full-length PRMT1 variant 1, deletion either the N-terminus or C-terminus of PRMT1 significantly decreased their protein stability (compare Lane 2, 3, 4 and 5 vs. Lane 6 in Fig. 22). In the same experiment, co-transfected Myc-tagged HSP70 could be used as the internal control to demonstrate that the expression efficiency of these vectors are at same level (Fig. 22 lower panel). Therefore, without the prerequisite to get equal amount of truncated proteins, the immunoprecipitation mapping assay could not be conducted.

The stability issue of PRMT1 protein has not been clearly studied before, but the structure analysis may provide some explanations. All PRMTs have a conserved core structure consisting of an AdoMet binding domain and a barrel-like domain (green and yellow domains in Fig. 21), which is about 310 amino acid long (85, 86). In PRMT1, there is a small dimerization arm domain (blue in Fig. 21) between the other two function domains, and homodimerization or homo-oligomerization of the PRMT1 subunit is important for its enzymatic activity (85). Besides the conserved 310 amino acids, PRMT1 can be produced up to seven protein isoforms, all varying in their N-terminal domain (87). The PRMT1 variant 1 used in my experiment is a relative long isoform that the N-terminal variable sequence is about 60-70 amino acids long. This may explain the observation that fragment 66-371 had a comparable expression level with full length protein (Lane 1 vs. Lane 6 in Fig. 22), because it contains nearly all the 310 amino acids long conserved structure. However, because all the conserved regions, an AdoMet binding domain, a dimerization

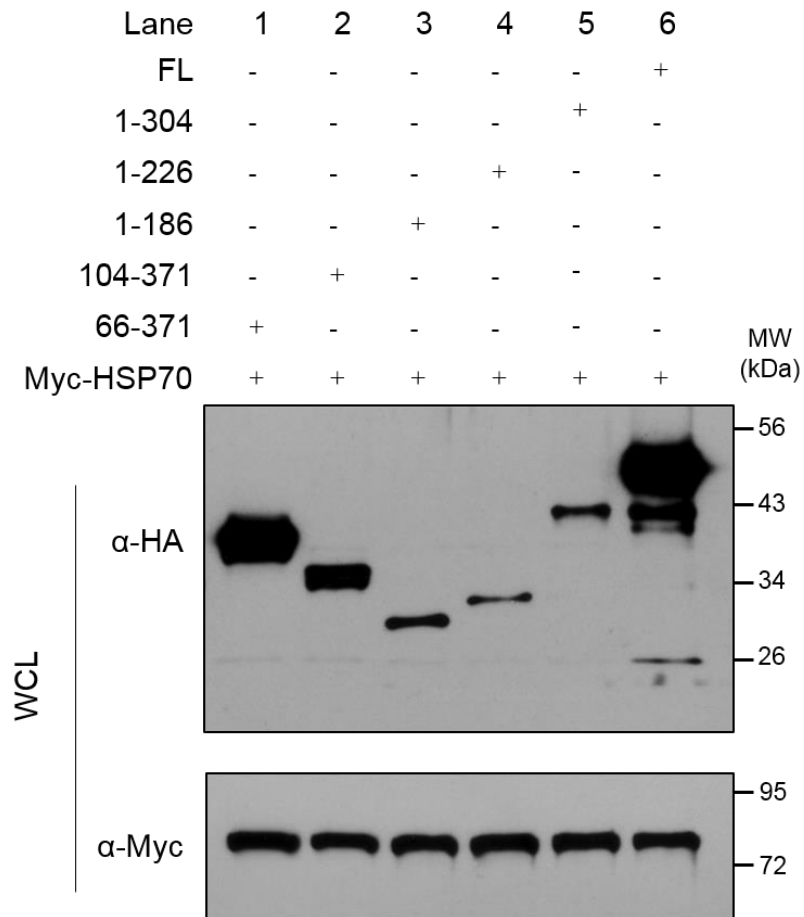
domain, and a barrel-like domain, fold together to form a single functional unit, deletions of virtually any portion of the conserved core structure will result in loss of structural integrity. Therefore, the truncated proteins will be quickly eliminated by cell intrinsic protein degradation system since they are regarded as improperly produced proteins by cells. This may explain why all other PRMT1 deletion mutants are less stable in 293T cells (compare Lane 2, 3, 4 and 5 vs. Lane 6 in Fig.22).



**Figure 21. Structure and deletion mutants of PRMT1 protein.**

(A) Structure of rat PRMT1. The colored domains are red for the variable N terminal helix, green for the AdoMet binding domain, yellow for the  $\beta$ -barrel structure, and blue for the dimerization arm. Adapted from Zhang X. and Cheng X., *Structure*, 2003,11, 509–520, with the permission from Cell Press.

(B) A series of human PRMT1 variant 1 deletion mutants used in following assays. The meaning of colored boxes is the same as the colored domains in (A). FL, full length



**Figure 22. Deletion mutants of human PRMT1 cannot be stably expressed in 293T cells.**

HA-tagged full-length (FL) or deletion mutants of human PRMT1 variant 1 and Myc-tagged HSP70 were co-transfected to 293T cells, and cell lysates were subjected to SDS-PAGE and then blotted with anti-Myc and anti-HA antibodies. WCL, whole cell lysate.

### **3.3.3 Two conserved arginine residues of HSP70 are methylated by PRMT1.**

Based on above protein-protein interaction analysis, I postulate that HSP70, as well as some other members in this protein family, may be a novel substrate of PRMT1. Glycine- and arginine-rich (GAR) sequence is the preferential methylation motif recognized by PRMT1, but protein sequence analysis failed to show any typical GAR motif in HSP70 protein. Therefore I planned to use mass spectrometry to *de novo* identify any possible arginine methylation in HSP70 protein. First, I used a specific antibody against asymmetric dimethylated arginine (ASYM24) to demonstrate the function of ectopically expressed PRMT1. Results showed that overexpression of PRMT1 could increase the overall methylation level of endogenous proteins, while knockdown of PRMT1 did the opposite (Fig. 23A). Particularly, the whole-membrane blotting also revealed that there were many proteins between 56 to 100 KDa that had arginine methylation modifications (Fig. 23A left panel), which may include HSP70.

To do the mass spectrometry analysis, Myc-tagged HSP70 and HA-tagged PRMT1 were co-transfected into 293T cells, and HSP70 protein was purified by immunoprecipitation with anti-Myc antibody conjugated to magnetic beads. Empty vector served as control. The prominent band corresponding to Myc-HSP70 was excised and subjected to mass spectrometry (Fig. 23B). Due to the trypsin digestion protocol we used and the amino acids composition of HSP70 protein, the mass spectrometry assay only covered about 70% peptide sequence of the whole HSP70 protein. The result indeed showed some methylated lysine and arginine residues, some of which were consistent with previous reports, meaning our assay was successful. The data analysis identified arginine 416 (R416) as a methylation site (Fig. 23C). This site is of great interest because it is located

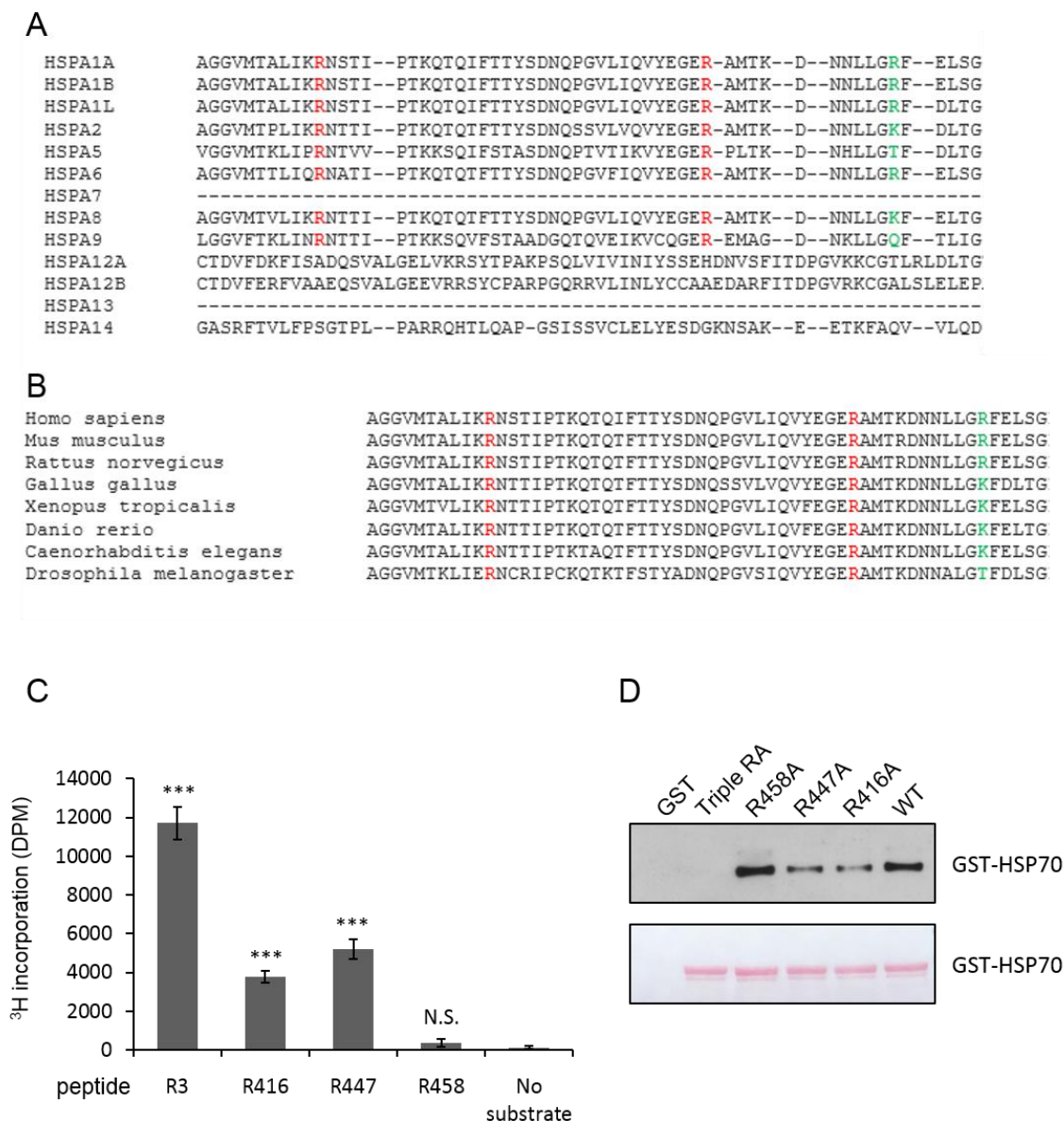


Protein sequence analysis showed that between amino acid 400-500, there are only four arginine residues (R416, R447, R458 and R469) in HSP70 protein, and arginine 469 has been proved to be methylated by CARM1/PRMT4 rather than PRMT1 in a recent study (62). Multiple sequences alignment revealed that among the remaining three arginine residues, two of them, R416 and R447, are much conserved and R458 is less conserved (Fig. 24). Particularly, R416 and R447 are not only highly conserved among different members of HSP70 family (Fig. 24A), but also conserved among various species during evolution history (Fig. 24B), suggesting that methylation on these residues may be functionally important. To verify whether these three arginine residues can be methylated by PRMT1, 20-amino-acids long peptides containing the three arginine residues (R416, R447 and R458), and the corresponding methylation site arginine-to-alanine (R-to-A) mutant peptides (R416A, R447A and R458A) were synthesized for the *in vitro* methylation assay. The mutant peptides were used as negative controls and a well-established 19-amino-acid peptide containing three GAR repeats (R3 in Fig. 24C) was used as a positive control (85). Results showed that when R416 and R447 peptides were incubated with purified PRMT1 and <sup>3</sup>H labeled S-Adenosyl methionine, the methyl group donor, there was a dramatic tritium incorporation detected by liquid scintillation counting (LSC). In contrast, R458 peptide failed to show such incorporation when compared with R458A mutant peptide (Fig. 24C).

To further confirm that arginine 416 and 447 can be methylated in the natural conformation of HSP70, three single R-to-A mutant (R416A, R447A and R458A) and a triple R-to-A mutant (triple RA) HSP70 expression vectors were constructed. GST-tagged wildtype and mutant HSP70 proteins were purified from bacteria and then used for *in vitro* methylation assay. Methylation of HSP70 was measured by autoradiography. As it is shown in Fig. 24D, wildtype HSP70 protein was methylated by PRMT1, and mutation either R416 or



R447 to alanine decreased about half of the methylation level, while mutation R458 to alanine did not affect the methylation level. If all three arginine residues were mutated to alanine, there was no methylation that could be detected by autoradiography. This observation suggests that R416 and R447 are the primary PRMT1 methylation sites of HSP70.



### 3.4 DISCUSSION

Emerging evidence suggest that PRMTs use both histone and non-histone proteins as substrates to modulate important biological processes during embryo development, cell growth and pathogenesis (17, 30, 89, 90). Methylation on both lysine and arginine residues of HSP70 protein has been known for decades, but the exact methylation sites, the methyltransferases involved in these reactions and the biological functions of such modifications are not clearly understood (91). Particularly, during the period of my dissertation study, the first evidence of arginine methylation (R469) of HSP70 mediated by PRMT4/CARM1 and its role in regulating gene activation was reported (62). However, in my present study, I provide evidence that two other highly conserved arginine residues of HSP70 are methylated by PRMT1, the founding member of type I PRMTs. Since both HSP70 and PRMT1 are evolutionally conserved proteins and can be found in nearly all eukaryotes, from yeast to human being, the PRMT1-mediated methylation of HSP70 may have a crucial role in life maintenance.

First, I used Co-IP assay to demonstrate that at least four members of HSP70 family, HSP70, HSC70, GRP75 and GRP78, could interact with PRMT1 both *in vitro* and *in vivo*. Although all of the proteins have similar structures, their functions are different due to their various subcellular localizations (57). Their interactions with PRMT1 not only suggest a broader substrate scope of PRMT1 but also indicate a conserved protein arginine methylation mechanism across HSP70 family members during evolution. But whether the modifications affect their functions in a similar way or different ways is an intriguing question that warrants further investigation.

Next, the interaction domain of HSP70 with PRMT1 was determined by using a series of HSP70 deletion mutant proteins. Result showed that amino acids 410 to 460 were essential for this protein-protein interaction. The following mass spectrometry and *in vitro*

methylation assay revealed two methylation sites located in the binding motif. Since this motif is the core part of substrate binding domain of HSP70, and several conserved arginine residues are predicted to facilitate either the intra-domain interactions of HSP70 protein or the binding of substrate peptides with HSP70 substrate binding pockets (92), methylation of these arginine residues may disrupt the hydrogen bonds required for such molecular interaction and thus change the binding activity of HSP70 to certain substrate peptides/proteins. For example, a recent study revealed that the amino acids 445 to 450 motif was responsible for the binding of HSP70 with superoxide dismutase 2 (SOD2) in the cytosol to transfer this enzyme to the mitochondria for activation (93). Whether this interaction is regulated by methylation of Arginine 447 remains further investigation.

## CHAPTER 4 ARGININE METHYLATION OF HSP70 REGULATES PANCREATIC CANCER DRUG RESISTANCE

### 4.1 INTRODUCTION

In Chapter 3, I demonstrated that HSP70 was a novel substrate of PRMT1 and two conserved arginine residues (R416 and R447) of HSP70 could be methylated by PRMT1 *in vitro* and *in vivo*. In this chapter, the biological consequences of these specific post-translational modifications will be studied. The question to be addressed is how HSP70 methylation will affect the biology of pancreatic cancer. Chemotherapeutic drug resistance is a commonly observed problem in pancreatic cancer treatment. Since HSPs are critical proteins involved in cell stress responses, and induced drug resistance is a typical cancer cell response under severe and continual stress, my study will focus on how arginine methylation of HSP70 regulates drug resistance in pancreatic cancer.

Gemcitabine is currently the first-line chemotherapeutic agent to treat pancreatic cancer. However, it only provides little benefit to prolong the survival and to moderately improve the quality of life of pancreatic cancer patients (94). Even combined with other chemo drugs, such as erlotinib or nab-paclitaxel, improvement in the survival of patients with advanced pancreatic cancer was still minimal, albeit statistically significant (16, 95). Gemcitabine is known to induce apoptosis of malignant cells after it is transported into cancer cells and phosphorylated by deoxycytidine kinase to become its active metabolite. But a high degree of inherent and acquired resistance to gemcitabine is often observed in clinical practice, which limits the efficiency of this chemotherapy (96). Overexpression of HSP70 in pancreatic cancers, as well as in some other cancer types, has been shown to confer drug resistance to gemcitabine (51, 97, 98), but the detailed mechanisms are not well understood.

Though PRMT1 plays important roles in various biological processes, its function in drug resistance is rarely studied. A research group demonstrated that PRMT1-mediated methylation of EGFR regulates acquired resistance to cetuximab, an EGRF targeting monoclonal antibody (99). Another group reported that PRMT1 may be a co-activator of pregnane x receptor in activating multidrug resistance protein 1 (MDR1) gene during acquired drug resistance in breast cancer cells (100). However, the role of PRMT1, particularly as a methyltransferase, in pancreatic cancer drug resistance has never been studied. Based on the evidence from last chapter, I postulate that PRMT1-mediated methylation of HSP70 protein is involved in the regulation of pancreatic cancer drug resistance.

In this chapter, first the HSP70 knock-out pancreatic cancer cells were generated. Then wildtype and methylation sites mutant HSP70 proteins were reconstituted into these cells to compare their different responses to gemcitabine treatment. In addition, their responses to other types of stress, like glucose deprivation, oxidative stress, etc., were also studied.

## 4.2 MATERIAL AND METHODS

### HSP70 and PRMT1 knock-out cell lines.

A CRISP-Cas 9 system was used to generate gene specific knock-out cell lines according to reference (101). Briefly, the first 100bp of coding sequences of human *HSPA1A/B* and *PRMT1* gene were analyzed for guide sequences design by an online CRISPR Design Tool (<http://crispr.mit.edu/>). The guide sequence for *HSPA1A/B* is forward: 5'-ATGGCCAAAGCCGCGGCGAT-3' and reverse: 5'-ATCGCCGCGGCTTTGGCCAT-3'. The guide sequence for *PRMT1* is forward: 5'-CATGATGCAGTTCGCGGCCT-3' and reverse: 5'-AGGCCGCGAACTGCATCATG-3'. The forward and reverse oligoes were annealed with each other and inserted into vector pSpCas9(BB)-2A-Puro (Addgene #62988), which was pre-digested by BbsI restriction endonuclease. The recombinant plasmid was transfected into MIA PaCa-2 cells and cells were treated with puromycin (1µg/ml) for 48-72 hours. After puromycin selection, single cell clones were seeded into 96-well plates by flow cytometry. After 10-14 day culture, each survival cell clone was tested for targeting gene knock-out by both Western blot and genomic DNA sequencing.

### In vitro cytotoxicity assay

A Cell Counting Kit-8 (CCK-8) method was used to measure the cytotoxicity of gemcitabine to different MIA PaCa-2 cell groups. Cells were seeded in 96-well plates (Corning Incorporated, Corning, NY, USA) at a density of 5,000 cells per well and incubated for 24 h in DMEM complete medium (Gibco, New York, NY, USA), then in medium containing 1-200 µM of gemcitabine for another 48 h. Vehicle treated control cells were included. Subsequently, culture medium was removed and fresh medium containing 10% CCK-8 reagent (Dojindo Molecular Technologies, Rockville, MD, USA) was added to each well, and the plates were incubated at 37°C for 1h. Then, the absorbance at 450 nm of each well was measured using a spectraMAX-M5 microplate reader (Molecular

Devices, Sunnyvale, CA, USA). All tests and analyses were performed in triplicate. Drug cytotoxicity was calculated as the fraction of reduced OD450 value after drug treatment to the OD 450 value of untreated cells. A graph of cytotoxicity versus concentrations of gemcitabine was plotted.

### **Glucose deprivation and gemcitabine treatment to cancer cells**

24h after transfection, control cell group was treated with gemcitabine diluted in high glucose DMEM medium (with 4500mg/L glucose, Sigma-Aldrich, St. Louis, MO, USA), while glucose deprivation cell group was treated with gemcitabine diluted in DMED medium without glucose and sodium pyruvate (Gibco, New York, NY, USA). After 48h culture, the cytotoxicity was measured as described above.

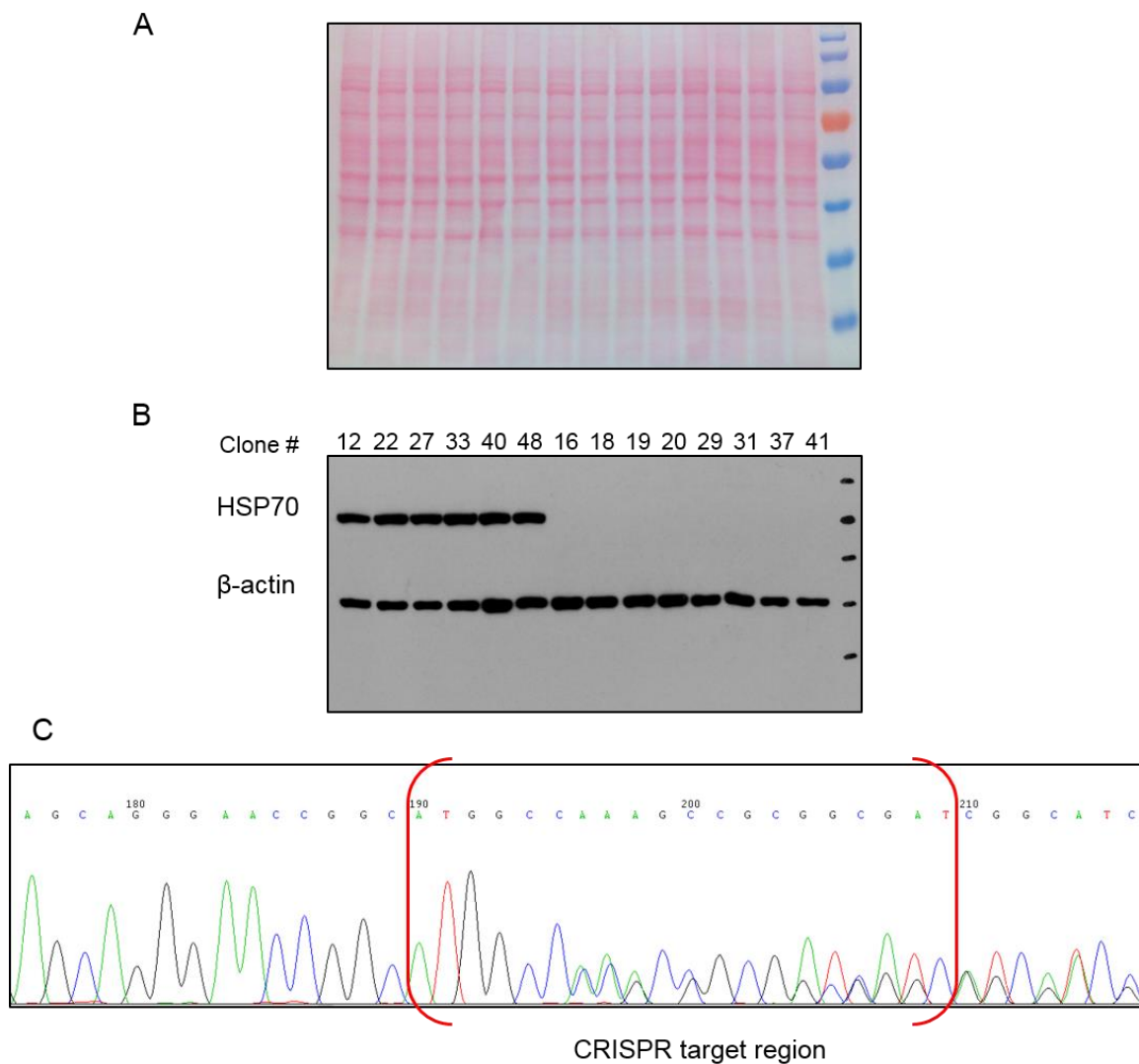


## 4.3 RESULTS

### 4.3.1 HSP70, but not PRMT1, knock-out cells are generated.

To study the biological functions of PRMT1-mediated HSP70 arginine methylation, first the HSP70 and PRMT1 knock-out cell lines were generated to delete endogenous proteins. A CRISPR/Cas 9 system was used to generate such cell lines. After single cell clone selection and culture, eight HSP70 knock-out MIA PaCa-2 cell clones were successfully generated (Fig. 25). Western blot showed that endogenous HSP70 protein was completely deleted (Fig. 25B) and DNA sequencing results demonstrated indel mutations in the CRISPR target region (Fig. 25C). Meanwhile, the HSP70 wildtype (WT) control cells were also generated by transfection of a scramble sgRNA followed by the same puromycin selection procedure.

In contrast, the generation of PRMT1 knock-out cell line was not successful. After single cell clone selection, Western blot and DNA sequencing results showed that in several cell clones, endogenous PRMT1 protein was indeed depleted (Fig. 26A and C), but these cells could not properly grow and proliferate. Extreme giant cells, about four to five times larger than normal cells, were observed in the first several days (yellow arrow in Fig. 26B IV), and then those giant cells became bubble-like structure and underwent apoptosis (red arrow in Fig. 26B IV). Finally, all PRMT1-depleted cell clones died in about 10 days.

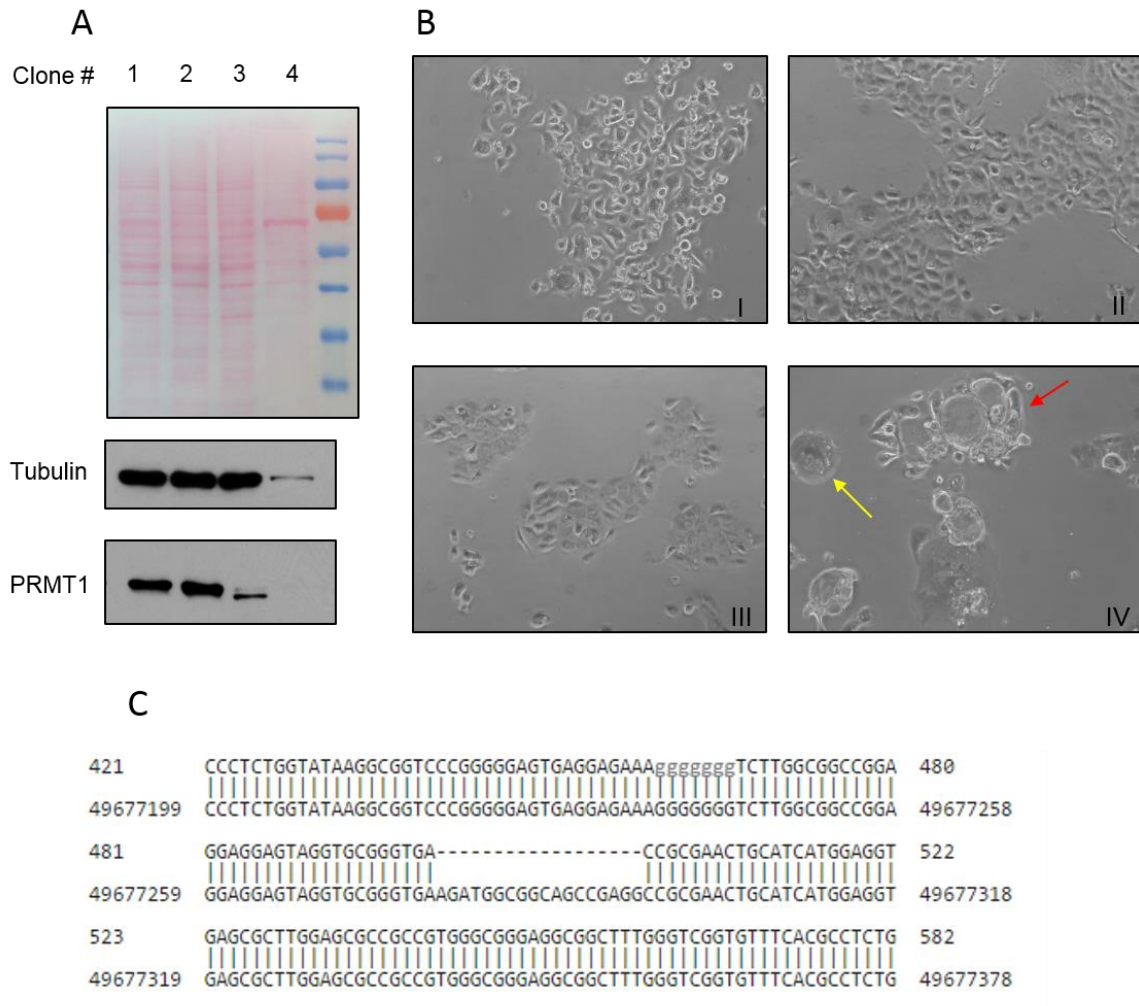


**Figure 25. HSP70 knock-out cell clones are generated.**

(A) Cell lysates from fourteen MIA PaCa-2 cell clones were subjected to SDS-PAGE and membrane was stained with Ponceau S.

(B) The membrane in (A) was blotted with anti-HSP70 and anti- $\beta$ -actin antibodies. HSP70 protein was deleted in eight cell clones.

(C) Sequencing result of clone #20. CRISPR target region revealed different indel mutations in two alleles indicated as dual peaks.



**Figure 26. PRMT1 knockout cell clones are not viable.**

(A) Cell lysate from four MIA PaCa-2 cell clones were subjected to SDS-PAGE and membrane was stained with Ponceau S. The membrane was blotted with anti-PRMT1 and anti-tubulin antibodies.

(B) Cell morphology of each clone in (A). Red arrow indicates a bubble-like cell structure and yellow arrow indicates a giant cell.

(C) The sequencing result of clone #4. 18bp fragment was deleted in both alleles of PRMT1 gene.

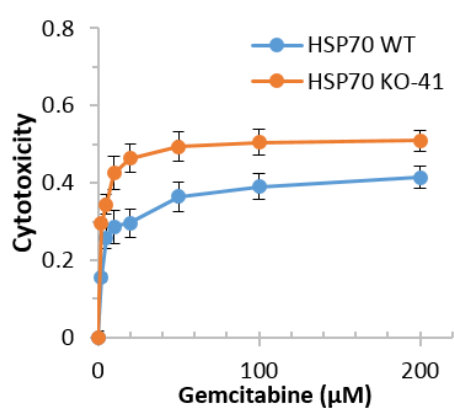
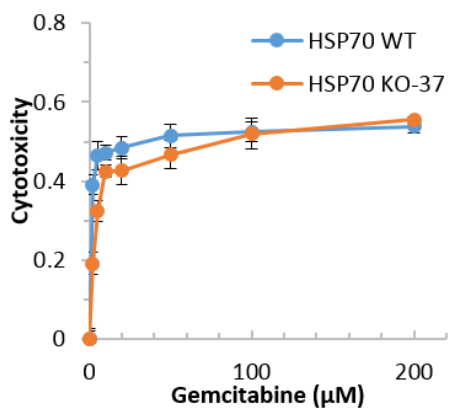
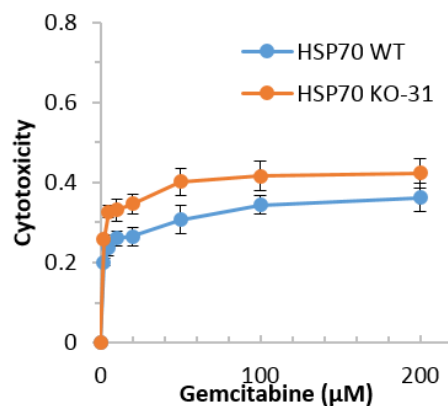
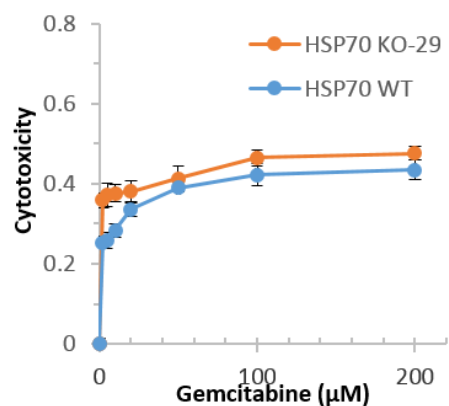
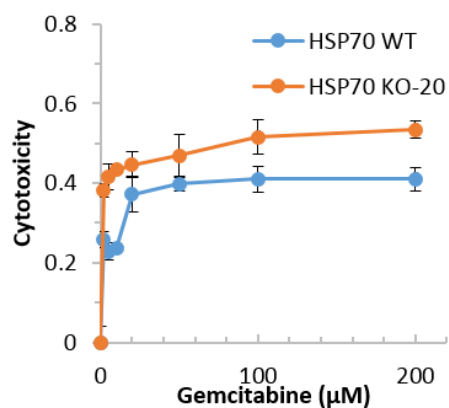
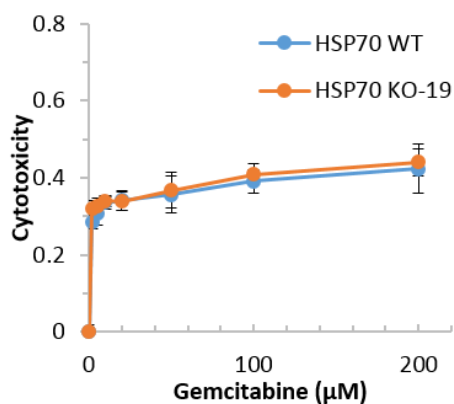
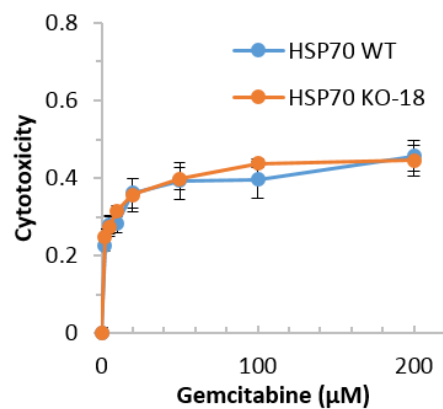
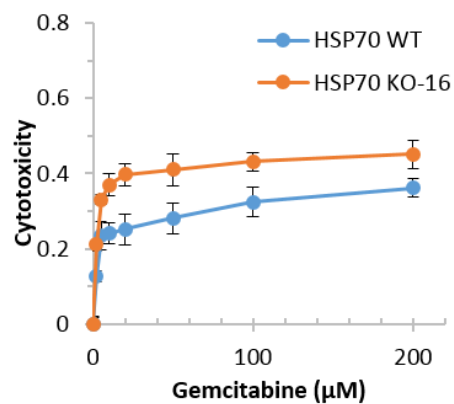
#### **4.3.2 HSP70 arginine methylation is essential for cancer cell drug resistance to gemcitabine.**

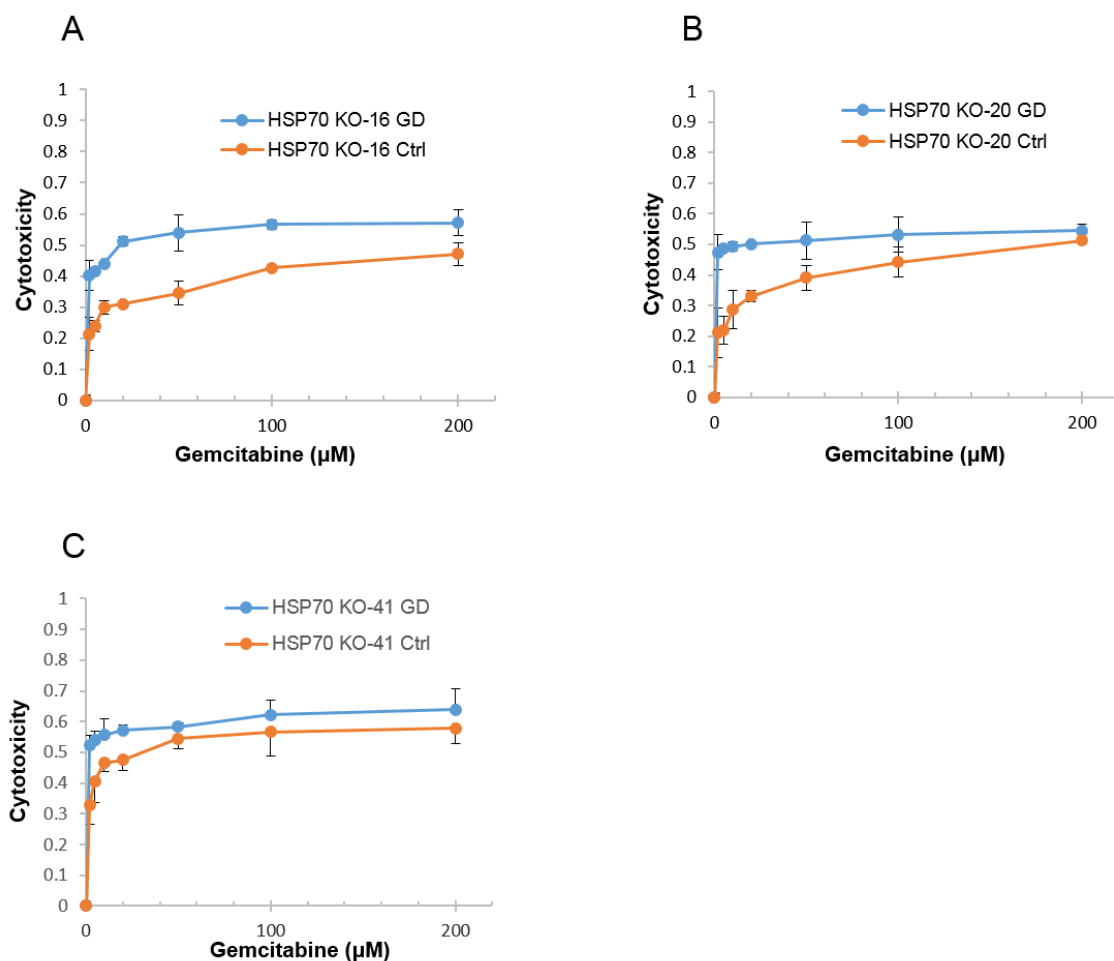
To study how HSP70 arginine methylation influences cancer cell drug resistance, first, I compared the cytotoxicity of gemcitabine to HSP70 wildtype (WT) and eight knock-out (KO) cell clones (Fig. 27). Results showed that in general, loss of HSP70 expression sensitized MIA PaCa-2 cells to gemcitabine. Five out of eight HSP70 knock-out cell clones showed increased cytotoxicity in a wide range of gemcitabine concentration (Fig. 27). This observation is consistent with previous studies and demonstrated the role of HSP70 in promoting chemotherapeutic drug resistance of cancer cells (51, 97).

Some members in heat shock protein 70 family, like GRP78 and GRP75, are glucose-regulated proteins. Their expression is induced by glucose shortage and is linked to multiple cellular processes (102). Similarly, HSP70 was also reported to play a role in the maintenance of normal cell physiology during glucose deprivation stress (103). Therefore I studied whether glucose level contributes to HSP70-mediated gemcitabine resistance. Results showed that glucose deprivation sensitized HSP70 knock-out MIA PaCa-2 cells to gemcitabine treatment, and this sensitization was more prominent at low drug concentration (Fig. 28).

#### **Figure 27. HSP70 depletion sensitizes MIA PaCa-2 cells to gemcitabine treatment.**

HSP70 wildtype (WT) MIA PaCa-2 cells and eight HSP70 knock-out (OK) cell clones were treated with gemcitabine at different concentrations for 24h. Relative cytotoxicity was measured using a CCK-8 method.

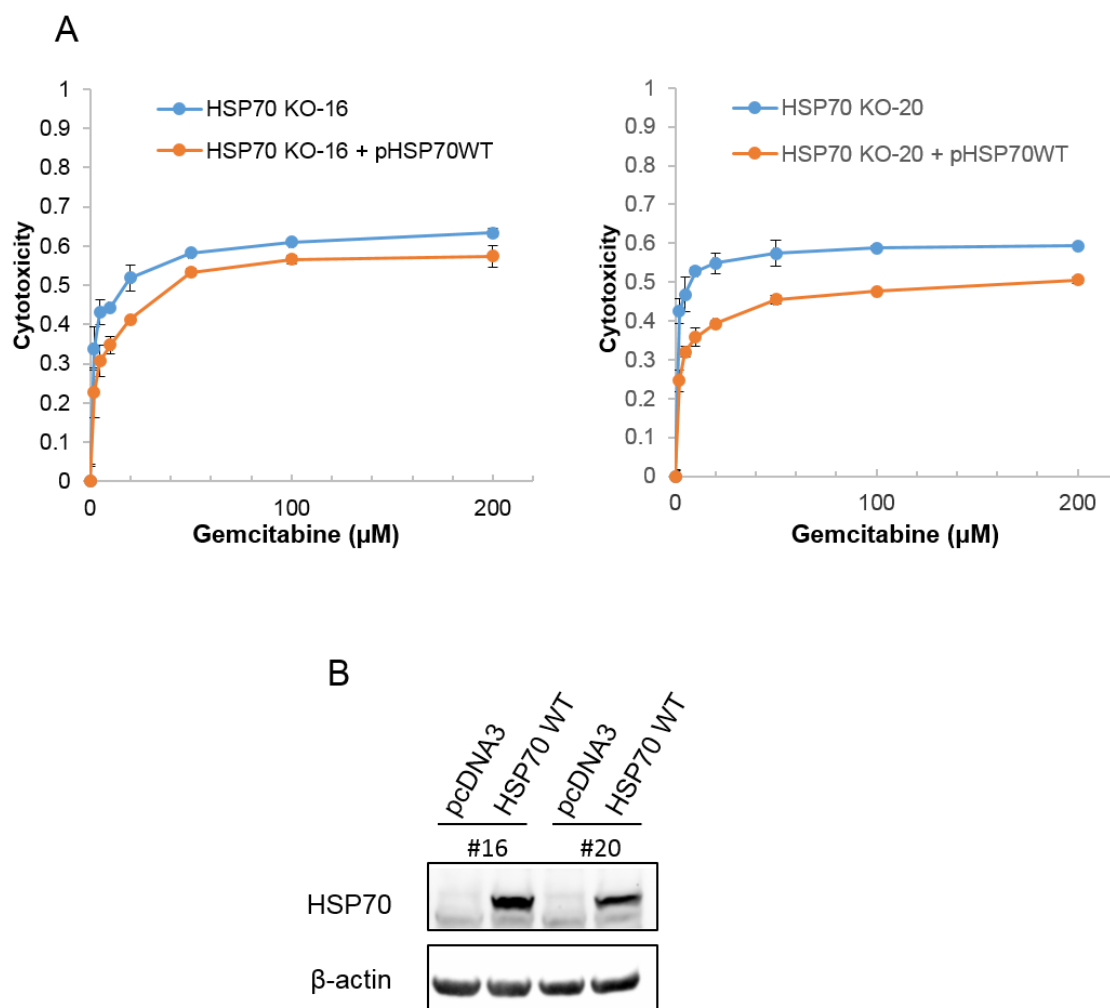




**Figure 28. Glucose deprivation sensitizes HSP70 knock-out MIA PaCa-2 cells to gemcitabine treatment.**

(A-C) Three HSP70 knock-out cell clones, KO-16, KO-20 and KO-41, were treated with gemcitabine at different concentration for 48h with or without glucose supplemented in culture medium. GD, glucose deprivation.

To test how arginine methylation influences HSP70-mediated drug resistance, first, the effects of wildtype HSP70 protein were validated in MIA PaCa-2 cells. Results showed that ectopic expression of wildtype HSP70 protein in HSP70 knock-out cells could restore the normal level of drug resistance to gemcitabine (Fig.29). Next, three single methylation site arginine-to-alanine mutant, R416A, R447A and R458A, and one triple methylation sites arginine-to-alanine mutant, 3Muts, were reconstituted into HSP70 knock-out cells, respectively. Relative cytotoxicity of gemcitabine to each cell group was measured and compared (Fig.30). Results showed that reconstitution of wildtype HSP70 rescued cell drug resistance to a similar level of normal MIA PaCa-2 cells. HSP70 R458A mutant could rescue most part of drug resistance when compared with wildtype HSP70. In contrast, R416A and R447A mutants could only rescue about half of the drug resistance level, and 3Muts HSP70 almost lost the ability to rescue drug resistance. Above evidence suggests that methylation of R416 and R447, rather than R458, is essential for drug resistance function of HSP70 in MIA PaCa-2 cells.

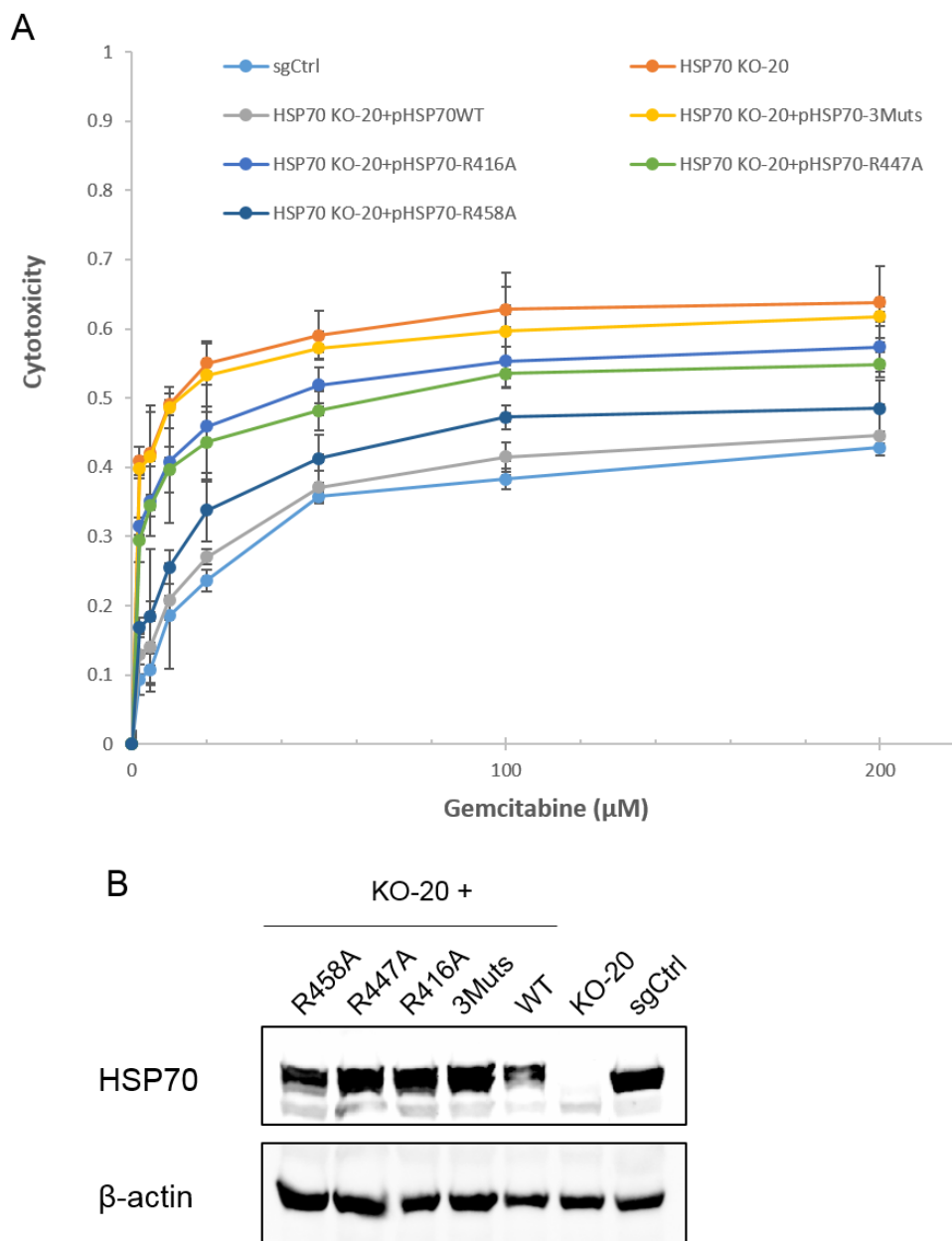


**Figure 29. Ectopic expression of wildtype HSP70 rescues drug resistance to gemcitabine in HSP70 knock-out cells.**

**(A)** Two HSP70 knock-out MIA PaCa-2 cell clones (KO-16 and KO-20) were transfected with pcDNA3 control plasmid or wildtype HSP70 expression plasmid. Cytotoxicity of gemcitabine to different cell groups was measured.

**(B)** Western blot of HSP70 protein in different cell groups.





**Figure 30. Methylation site arginine-to-alanine (R-to-A) mutant HSP70 protein lost drug resistance function to gemcitabine in MIA PaCa-2 cells.**

**(A)** HSP70 knock-out MIA PaCa-2 cells (KO-20) were transfected with wildtype, single methylation site or triple site R-to-A mutant HSP70 expression plasmids. Cytotoxicity of gemcitabine to different cell groups was measured. sgCtrl cells were used as control group.

**(B)** Western blot of HSP70 protein in different cell groups.

#### 4.4 DISCUSSION

In this chapter, I demonstrated that arginine methylation of R416 and R447 were essential for HSP70-mediated drug resistance against gemcitabine by using HSP70 knock-out cells and wildtype and a series of methylation site mutant HSP70 proteins. Several studies have shown that overexpressed HSP70 protein increased drug resistance against gemcitabine in multiple cancer cells, and the mechanism was associated with cell apoptosis and/or autophagy (51, 97, 98). All of these studies mainly focused on the expression level of HSP70 protein, and used a bioflavonoid, quercetin, to suppress HSP70 expression and therefore to sensitize cancer cells to gemcitabine treatment. However, one of these studies (97) and my study found that gemcitabine treatment alone did not affect HSP70 expression level (data not shown), which means that HSP70-mediated drug resistance may not be achieved by increasing HSP70 expression level, but changing its chaperone activity. If it is the case, post-translational modifications (PTMs) of HSP70 would be a possible way to quickly regulate HSP70 chaperone activity under stress conditions.

The binding of HSP70s to their client proteins is determined by the conformation of the C-terminal lid domain, which in turn is regulated by nucleotide binding and hydrolysis in the N-terminal nucleotide binding domain. Therefore, PTMs of HSP70 regulate its chaperone activity mainly by two mechanisms, changing its ATPase enzymatic activity and/or affecting its binding affinity to client proteins. Some examples include discoveries that acetylation at lysine 77 (K77) of HSP70 increased its ATPase enzymatic activity (104), and methylation at lysine 561 (K561) of HSC70 reduced its affinity to a client protein,  $\alpha$ -synuclein ( $\alpha$ -Syn) (61). In addition, a recent study also showed that methylation at arginine 469 (R469) of HSP70 was involved in TFIIH recruitment, its association with chromatin,

and gene transcription, which was distinct from its “classic” function as molecular chaperone (62).

In my research, the molecular mechanisms how methylation of R416 and R447 influences HSP70 function in drug resistance await for further study. By comparing the ADP-bound and ATP-bound crystal structures of DnaK, the human HSP70 homologue in *E. coli*, I found that R416 residue is located in the interface between the NBD and SBD of the ATP-bound HSP70 (data not shown). Because the interaction of NBD with SBD is critical for the allosteric activation of HSP70 (105-107), the putative location of R416 may suggest its role in regulating ATPase activity of HSP70. In another hand, both R416 and R447 residues are located in the SBD domain of HSP70, thus they may be the contacting residues between HSP70 and certain client peptides/proteins. Indeed, a recent paper showed that arginine 447 was important for the binding of HSP70 with superoxide dismutase 2 (SOD2), and this interaction regulated SOD2 antioxidant function in a stress-inducible way (93). Whether methylation at these two arginine residues will affect the binding of HSP70 to its client proteins, particularly proteins that are involved in drug resistance, warrants further study.

## CHAPTER 5 SUMMARY, DISCUSSION AND FUTURE DIRECTIONS

### 5.1 SUMMARY

#### 5.1.1 Pro-tumorigenic role of PRMT1 in pancreatic cancer

In this dissertation, I demonstrated that the level of PRMT1 expression was elevated in both human pancreatic tumor tissues and mutant *Kras*-induced mouse pancreatic tumors. Moreover, the IHC staining results from mouse model also revealed that the expression of PRMT1 protein was gradually elevated during pancreatic cancer progression, from low grade PanIN to high grade PanIN and finally PDAC. This observation also prompted an intriguing question that how PRMT1 expression is dysregulated during pancreatic carcinogenesis. My functional study further demonstrated a pro-tumorigenic function of PRMT1. Overexpression of PRMT1 promoted cancer cell proliferation, migration and invasion *in vitro* and xenograft tumor growth *in vivo*, while inhibiting PRMT1 expression by siRNA did the opposite. Besides, the effects of PRMT1 specific small molecular inhibitors were tested on both human and mouse pancreatic cancer cells. They could induce dramatic cell apoptosis *in vitro* and decrease tumor growth *in vivo*. In summary, many pieces of evidence in this dissertation suggest a pro-tumorigenic role of PRMT1 in pancreatic cancer pathogenesis and inhibition of PRMT1 expression and/or enzymatic activity can decrease pancreatic cancer growth.

#### 5.1.2 HSP70 protein is a novel methylation substrate of PRMT1

In my study, I found that four major members of HSP70 protein family were interacting partners of PRMT1 in protein-protein interaction study. Due to the protein structure and sequence similarity among these proteins, it prompted me to study whether there is a conserved structure domain of these proteins that can interact with and be methylated by PRMT1. Using Co-IP assay, I demonstrated that at least four HSP70 family members, HSP70, HSC70, GRP75 and GRP78, could interact with PRMT1 *in vitro*. HSP70 was

selected as a representative protein for following studies. Further Co-IP and GST pull-down assays with a series of HSP70 deletion mutants revealed amino acids 410-460 as a conserved interacting domain with PRMT1, and bioinformatics analysis indicated that there are three arginine residues that could be potential PRMT1 methylation sites. Then *in vitro* methylation assay was performed using synthesized peptides and recombinant proteins as substrates. Results showed that two conserved arginine residues, R416 and R447, of HSP70 were the primary PRMT1 methylation sites. The mass spectrometry analysis also confirmed the existence of protein arginine methylation in pancreatic cancer cells.

### **5.1.3 Arginine methylation is essential for HSP70-mediated cell stress responses**

Since HSP70 is a critical protein involved in multiple cell stress responses, and chemotherapeutic drug resistance is a common problem in clinical practice, how arginine methylation influences HSP70 function in drug resistance is studied in my dissertation. Using HSP70 knock-out cell line, wildtype and methylation sites mutant HSP70 recombinant proteins, my data demonstrated that arginine methylation at R416 and R447 were essential for HSP70-mediated drug resistance to gemcitabine. Mutation of arginine to alanine at these positions prevented PRMT1-mediated methylation and therefore decreased cell drug resistance to gemcitabine both *in vitro* and *in vivo*. In contrast, reconstitution of wildtype HSP70 protein could rescue the drug resistance of HSP70 null cells. Meanwhile, the arginine methylation of HSP70 were also proved to be important for other types of environmental stress, like hydrogen peroxide induced oxidative stress.

## 5.2 DISCUSSION AND FUTURE DIRECTIONS

### 5.2.1 Study the mechanisms underlying PRMT1 overexpression during pancreatic carcinogenesis

My study demonstrated that PRMT1 protein is overexpressed in several gastrointestinal cancer types, such as pancreatic cancer, gastric cancer and colon cancer. Moreover, IHC staining results from mouse pancreatic cancer model also revealed that during pancreatic carcinogenesis, PRMT1 expression is gradually elevated. Therefore, how PRMT1 is overexpressed during this process is a question awaiting for answers. Analysis of TCGA data shows that copy number change and somatic mutations of *PRMT1* gene are rarely detected in human tumor samples (data not shown), thus the transcriptional and/or post-transcriptional regulation should be possible reasons for PRMT1 overexpression.

First, the results from another study using pulmonary inflammation model showed that IL-4 increased PRMT1 expression through STAT6 signaling in airway epithelial cells, while IL-1 $\beta$  up-regulated PRMT1 through NF- $\kappa$ B pathway in airway fibroblasts in lung (108). Similarly, in my preliminary study, I found that TNF- $\alpha$  treatment could induce PRMT1 expression in several pancreatic cancer cell lines and 293T cells (data not shown). Since pancreatic cancer, including its pre-cancerous lesions, like PanIN, is usually associated with tissue inflammation, whether inflammatory molecules and inflammation signaling pathways play a role in PRMT1 up-regulation warrant further study.

Second, during the stepwise progression of PanIN from low grade to high grade in stage 1, 2, and 3, different genetic alterations are accumulated (1). *KRAS* mutations are detected in more than 90% of PanIN cases of all grades, and the average concentration of mutant *KRAS* alleles increase significantly with increasing grade of PanIN (109). In addition, the inactivation mutation of *CDKN2A/p16*, *p53*, and *SMAD4* tumor suppressors is detected with increasing frequency in stage 2 and stage 3 lesions of PanIN (1). Thus

the increasing genetic alternations, particular *KRAS* mutation, can be a reason for increasing PRMT1 expression during pathogenesis.

Third, other regulatory mechanisms, such as microRNA, could also be involved in PRMT1 dysregulation. A recent study showed that in human hepatocellular carcinoma, miR-503 suppressed cancer metastasis by targeting PRMT1 (71). Therefore the microRNA regulation is another research direction in my future study.

### **5.2.2 Study how arginine methylation influences HSP70 chaperone function**

In my dissertation, I demonstrated that arginine methylation at R416 and R447 of HSP70 were essential for its drug resistance function. However, how this is achieved at the molecular level and whether this is due to the alternation of HSP70 chaperone function is not clearly understood.

As it is stated in Chapter 1 that addition of methyl group(s) to an arginine residue does not change the positive charge of this residue, but removes a potential hydrogen bond donor of this residue. The methylation sites R416 and R447 identified in my study are located in the substrate binding domain of HSP70. Methylation of these residues may affect the binding affinity of HSP70 to certain client proteins and/or co-chaperone proteins, and therefore lead to the observed cell behavioral change under chemo drug treatment.

For example, a recent study reported that the lysine acetylation at K77 of HSP70 promoted its binding to a co-chaperone Hop, and deacetylation at that site switched its binding preference to another co-chaperone CHIP (104). Whether the arginine methylation discovered in my study works in a similar way is to be addressed. Another research showed that glutamic acid 446 and arginine 447 of HSP70 were critical for the regulation of superoxide dismutase 2 (SOD2) antioxidant function (93). Considering my data proved

that arginine 447 was a methylation site of PRMT1, it is plausible that methylation at this specific position is important for the binding of HSP70 with SOD2.

HSP70 protein has an ATPase enzyme activity, and ATP/ADP binding-induced conformational change is an important regulatory mechanism for HSP70 allosteric activation as a protein chaperone. Protein structure simulation and analysis in a recent study revealed that arginine 416 is located in the interface between the NBD and SBD of the ATP-bound conformation of HSP70 (104). Thus whether methylation at this amino acid residual will regulate the ATPase cycle of HSP70 needs further study.

### **5.2.3 Study combined therapy of PRMT1 inhibitor and gemcitabine to treat pancreatic cancer.**

In this dissertation, I demonstrated the pro-tumorigenic function of PRMT1 in pancreatic cancer development, and that PRMT1-mediated arginine methylation was important for HSP70-mediated gemcitabine resistance. These observations provide at least two rationales to test combination use of PRMT1 inhibitor and gemcitabine to treat pancreatic cancer. First, inhibition of PRMT1 enzymatic activity itself can reduce tumor growth. This is not only proved in Chapter 2 of my dissertation, but also shown in another study using the same inhibitor (DB75) to reduce leukemia cell growth (69). Second, inhibition of PRMT1 activity can potentiate cytotoxicity of gemcitabine to cancer cells by reducing cell drug resistance. A recent study revealed that PRMT1-mediated Gli1 methylation promoted drug resistance function of Gli1 (110). This discovery, together with the function of HSP70 methylation proved in my dissertation, suggests that PRMT1 plays a critical role in mediating chemo drug resistance, and therefore inhibition of PRMT1 in cancer cells may alleviate drug resistance. Furthermore, the PRMT1 specific inhibitor (DB75) used in my study, is originally developed and utilized for anti-parasite treatment by targeting the minor groove of AT-rich DNAs (111), thus the biological safety of this drug should not be a



concern in expanding its application to cancer therapy. One thing needs to be considered is the potential damage of this drug to normal pancreatic and other somatic cells. In my future study, I will study the tissue or cell selectivity of PRMT1 inhibitor to determine whether or not it only induces apoptosis of cancer cells rather than normal cells.

#### **5.2.4 Study the survival dependency on PRMT1 of pancreatic cancer cells**

In Chapter 4 of my dissertation, I showed that the PRMT1 knock-out MIA PaCa-2 pancreatic cancer cells could not properly grow and proliferate. This observation is of great interest because it may indicate that pancreatic cancer cells have survival dependency on PRMT1. An early paper showed that PRMT1 null mouse embryos died at embryonic day 6.5, but the embryonic stem (ES) cell lines established from PRMT1 null blastocysts were viable (112). Later on, another study revealed an essential role of PRMT1 in genome maintenance and cell proliferation using Cre-loxP mediated PRMT1 conditional knock-out mouse embryonic fibroblasts (MEFs) (29). Their data showed that loss of PRMT1 in MEFs leads to a higher incidence of chromosome losses, gains, structural rearrangements, and polyploidy, and finally resulted in cell death (29). This observation is similar to my data, thus I postulate that the extreme giant cells formed by PRMT1 null MIA PaCa-2 cells in my study may be a result of genome instability, aneuploidy and defect in cell division.

During the preparation of this dissertation, a study published by Hsu et al. reported that Prmt1 is required for tumor initiation in p53/Rb-null osteosarcomas (OS) mouse model (113). Moreover, when Cre recombinase was induced in Prmt1<sup>flox/flox</sup> OS cells, those cells underwent Prmt1 inactivation and growth arrest *in vitro* (113). This observation also supports an essential role of PRMT1 in cancer cell growth and/or survival, although those authors used another cancer type that was different from pancreatic cancer in genetic background.

To study the physiological role of PRMT1 in pancreas development and pathogenesis, a pancreas specific PRMT1 conditional knock-out mouse model should be generated and utilized. Deletion of PRMT1 protein at different developmental stages can show the dependency of PRMT1 in normal pancreas development. When crossed with pancreas specific *Kras* mutant mouse line, the resultant mouse line can be used to study the role of PRMT1 in pancreatic cancer development. For example, it is interesting to assess whether deletion of PRMT1 in *Kras* mutation background can delay or even prevent PDAC initiation and development.

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