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## TARGETED INHIBITION OF SP1 TRANSCRIPTION FACTOR AND ANTI-ANGIOGENESIS OF HUMAN PANCREATIC CANCER

Zhiliang Jia

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# **TARGETED INHIBITION OF SP1 TRANSCRIPTION FACTOR AND ANTI-ANGIOGENESIS OF HUMAN PANCREATIC CANCER**

by

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**TARGETED INHIBITION OF SP1 TRANSCRIPTION FACTOR AND  
ANTI-ANGIOGENESIS OF HUMAN PANCREATIC CANCER**

**A**

**DISSERTATION**

**Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
and  
The University of Texas  
M. D. Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment**

**of the Requirements**

**for the Degree of**

**DOCTOR OF PHILOSOPHY**

**by**

**Zhiliang Jia, M.S.**

**Houston, Texas**

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# **Targeted Inhibition of Sp1 Transcription Factor and Anti-angiogenesis of Human Pancreatic Cancer**

**Publication No.**\_\_\_\_\_

**Zhiliang Jia, Ph.D.**

**Supervisory Professor: Keping Xie, M.D., Ph.D.**

Transcription factor Specificity Protein 1(Sp1) is reported to be essential for vascular endothelial growth factor (VEGF) constitutive expression in human pancreatic adenocarcinoma. The definitive role of Sp1 in angiogenesis, the impact of anti-angiogenic therapy on the Sp1/ VEGF signaling and the Sp1 signaling alteration on the anti-angiogenic therapy effect are unclear. The understanding of Sp1 regulation on VEGF and their interactions has significant clinical implications.

Sp1 and VEGF expression and microvessel density (MVD) were analyzed using pancreatic cancer patients specimens through immunohistochemistry staining. The impact of Sp1 expression alternation on angiogenesis and tumor progression in nude mice were determined by knockdown Sp1 with small-interfering RNA (siRNA). Sp1 protein expression was correlated with the MVD ( $P < 0.001$ ) and VEGF expression ( $P < 0.05$ ). In mouse models, tumor progression and metastasis were inhibited after knockdown of Sp1 expression. The antitumor

activity was correlated with the down-regulation of Sp1 downstream angiogenic factors caused by Sp1 knockdown.

Sp1 and its downstream angiogenic genes expression were suppressed by mithramycin treatment both *in vitro* and *in vivo*. Moreover, mithramycin treatment reduced MVD *in vivo*. This was consistent with the down-regulation of VEGF, PDGF, and EGFR. Human xenograft pancreatic tumor growth was suppressed by Bevacizumab treatment. Both western blot and immunohistochemistry staining revealed that Sp1 and its downstream angiogenic genes expression were up-regulated by Bevacizumab treatment. Bevacizumab and mithramycin combination treatment synergistically suppressed tumor growth *in vivo*. This is correlated with the down-regulation of Sp1 and its downstream angiogenic genes expression. Bevacizumab treatment may trigger a positive feedback to up-regulate angiogenic factors through Sp1 trans-activation and this mechanism can be diminished by mithramycin treatment.

Combination treatment of mithramycin and tolfenamic acid which has been shown to facilitate Sp1 protein degradation had synergistic cell growth inhibition effect *in vitro*. *In vivo*, metronomic low-dose combined treatment of mithramycin and tolfenamic acid produced tumor suppression in mouse model. Gene expression analysis showed that the combination treatment synergistic down-regulated Sp1 and its downstream angiogenic molecule VEGF.

In summary, experimental results and clinical research suggested that Sp1 signaling is very important for angiogenesis of pancreatic cancer. Mithramycin treatment down-regulated Sp1 protein through interpose its transcription and decreased Sp1 downstream angiogenic genes. These down-regulations were correlated with the antitumor activity. Synergistic down-regulation of Sp1 and decreased expression of its downstream angiogenic molecules in turn significant reduced the angiogenic potential of pancreatic cancer cells and is an effective anti-angiogenesis strategy. Therefore, this study showed that Sp1 is a key factor of angiogenesis and manipulation of Sp1/VEGF signaling has clinical implication in anti-angiogenic therapy of pancreatic cancer.

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## ABBREVIATIONS

9aaTAD	nine-amino-acid transcriptional activation domain
Btd element	Buttonhead element
BVZ	Bevacizumab
ChIP	Chromatin immunoprecipitation
CRADA	cooperative research and development agreement
EGFR	Epidermal growth factor receptor
FDA	Food and Drug Administration
FGF	fibroblast growth factor
Flt-1	fms-like tyrosine kinase 1
Flt-4	fms-like tyrosine kinase 4
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HGF	hepatocyte growth factor
HIF	hypoxia inducible factor
HUVECs	human umbilical vein endothelial cells
IGF-1	insulin-like growth factor-I
IGF-1R	insulin-like growth factor-I receptor
IPMN	intraductal papillary mucinous neoplasm
KDR	kinase insert domain receptor
MCN	mucinous cystic neoplasm
MIT	Mithramycin



MTT	methylthiazolyldiphenyl-tetrazolium bromide
MVD	Microvessel density
NCI	National Cancer Institute
NF- $\kappa$ B	nuclear factor $\kappa$ B
NIH	National Institutes of Health
NRP-1	Neuropilin-1
NRP-2	Neuropilin-2
OS	overall survival
PanIN	Pancreatic intraepithelial neoplasm
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PIGF	placenta growth factor
PLGA	Poly lactide co-glycoide
siRNA	Small-interfering ribonucleic acid
Sp1	Specificity protein 1
TA	Tolfenamic acid
TAD	transcriptional activation domain
TBP	TATA-binding protein
TFIID	Transcription factor II D
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

## **BACKGROUND**

### **PANCREATIC CANCER IS A LETHAL DISEASE**

The pancreas is an organ of the digestive system. It has two major functions that are performed by its two components respectively. The exocrine pancreas, which consists of ductal and acinar elements and is about 80 percent of the organ, produces several enzymes that are secreted and can digest food in intestine; and the endocrine pancreas, which mainly consists of islets and is about 20 percent of the organ, makes hormones that can enter circulating system and have different functions [1]. Pancreas tumors may occur in exocrine part of pancreas (which are the classic pancreatic adenocarcinoma) or in endocrine part of pancreas.

Pancreatic cancer is the eighth cause of death and the thirteenth incidence cancer worldwide [2, 3]. In United State, it is currently the fourth leading cause of cancer related deaths [3]. According to the American Cancer Society, there were about 42,470 new pancreatic cancer cases and about 35,240 people die from the disease in 2009. Age is the most important predictor of pancreatic cancer. It is rare before 45 and the incidence rises greatly after that [4]. The male to female ratio is 1.3:1. The number one risk factor of pancreatic cancer is smoking and diet is the second risk factor associated with this disease. Consumption of animal protein and fat is associated with increased risk and vegetable diet is associated with decreased risk [2, 4, 5]. Several medical conditions including diabetes,

pancreatitis, gastrectomy and cholecystectomy are also associated with increased risk of pancreatic cancer [6-12].

Because of the non-specificity and late appearance of symptom, pancreatic cancer is difficult in diagnosis. It is an aggressive type of tumor and the systemic treatments availability is limited. The five-year survival rate of pancreatic cancer patients is only about 5 percent and median survival duration is about 4 to 6 months (from diagnosis to death). Most people were diagnosed at advanced stages [3, 13, 14]. It is highly resistant to chemotherapy and radiotherapy. The pancreatic cancer cells are highly metastasis *in vivo*, mostly to liver and lung. Lots of angiogenesis and invasion key regulatory molecules are reported to be aberrantly expressed [15, 16].

Most of the pancreatic tumors are primary pancreas tumors including exocrine tumor, endocrine tumor and non-epithelial tumors. Pancreatic tumor that metastasizes from other organ is about 3 to 11 percent [17-19]. In pancreatic carcinoma, ductal adenocarcinoma is about 80 to 85 percent and 90 percent if the variants are included [20, 21]. The variants of ductal carcinoma may include adenosquamous carcinoma, which has squamous component; mucinous noncystic carcinoma, which has well differentiated glands that consists of more than 50 percent of mucinous tissue; signet-ring cell carcinoma, which consists of a dyshesive population of malignant cells infiltrating the pancreas and has cytoplasmic mucin vacuoles that compress the nucleus and is similar to signet-ring cell carcinoma of stomach; and several other rare variants [22-24]. Besides

ductal adenocarcinoma, 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. Pancreatic endocrine tumors are only 0.5 to 4 percent of all pancreatic primary tumors. Most of the pancreatic endocrine tumor occurs in adult people. It mainly includes insulinoma, small cell carcinoma, glucagonoma, gastrinoma and so on. Non-epithelial tumors have 2 types, soft tissue tumor and lymphoma. This dissertation focuses on pancreatic ductal adenocarcinoma angiogenesis and experimental therapeutics. The word pancreatic cancer in this dissertation was assumed to be pancreatic ductal adenocarcinoma without specific explanation after this point.

Generally pancreatic cancer appears at pancreas head. It may infiltrate to adjacent tissue and metastasize to distance organ such as liver and lung. Based on its histological and cytological characteristics, it seems that pancreatic cancer is developed from pancreas ductal cell. But research showed each type of cells of pancreas has the capacity to dedifferentiate the duct like phenotype [25, 26]. Genetic engineered mouse models were developed and expected to give a clear cut answer to the cell origin of pancreatic cancer and beyond [26-29]. Besides this question, researches also focus on the pancreatic ductal adenocarcinoma precursors. Pancreatic intraepithelial neoplasm (PanIN), mucinous cystic

neoplasm (MCN) and intraductal papillary mucinous neoplasm (IPMN) were identified as pancreatic cancer precursor lesions [30-32]. PanIN that could be found in the pancreas smaller caliber ducts is the most extensively studied one. A new nomenclature for classification of duct lesion in pancreas was documented in 1999 and published 2001 [33]. In addition to the histological and morphological characters, molecular genetics changes of PanIN development were also documented [33, 34]. K-Ras mutation was found to be the key molecular event during the process [26, 35]. Among the complicated signaling network evolved in PanIN, a small class of molecules, such as p53 and nuclear factor  $\kappa$ B (NF- $\kappa$ B), control the neck, which is the messenger RNA transcription, of the whole network. Genetic and epigenetic changed molecules and tumor environmental factors, such as hypoxia, acidosis and so on, directly or un-directly use one or more transcription factors to modulate tumor cell physiological activities.

## TRANSCRIPTION FACTOR AND CANCER BIOLOGY

Transcription factors are protein complexes that are responsible for regulating gene expression in response to upstream signal [36, 37]. The regulation links *cis*-acting DNA sequence and the corresponding gene expression and may be activation or repression. Transcriptional activation facilitates RNA polymerase binding to *cis*-acting DNA sequence. Transcriptional repression includes the global or local level interference with the transcription genes directly or indirectly. The following discussion focuses on transcriptional activation and transcription factor is supposed to be transcription activator without specific explanation.

In the genome of life, each gene has a DNA sequence called promoter. It can control transcription of the gene. Promoter is usually located right before the coding region and its length may vary in different genes. RNA polymerase needs to bind to promoter before the beginning of gene transcription [38, 39]. Protein sequence information in the gene can be transcribed into messenger RNA by RNA polymerase [40]. RNA polymerase II is mainly responsible for messenger RNA transcription. Protein synthesis of the gene is then directed by the messenger RNA that is carrying the information [41]. Transcription factors can regulate gene transcription by controlling RNA polymerase's access to the promoter region. To fulfill this function, transcription factors have both *cis*-element recognition and transcriptional activation. Generally these two functions are fulfilled by different regions of the protein primary structure.

To fulfill the functions, transcription factors must recruit themselves on promoter through interaction with specific DNA sequences. Generally each kind of transcription factor owns one and only one handful structural motifs. The motif that can bind to promoter is called DNA binding domain. (A protein motif is the small spatial part of the whole molecule.) Transcription factor may interact with basal factors or other proteins called co-factor by other portions. The DNA-binding domain of a transcription factor recognizes *cis*-element [38]. In eukaryotic life form, most genes' promoter has the "TATAA" sequence, called the "TATA box". It is usually located in twenty-five to thirty nucleotides before the transcription start site. Transcription factors that bind this sequence are called

TATA-binding protein (TBP). TBP is one of the most important transcription factors and the main component of general transcription factor TFIID [38, 42, 43]. Chemically, transcription factors bind *cis*-element using a combination of electrostatic and Van der Waals forces. Transcription factors' bindings to promoter are sequence specific, because the combination of the chemical interactions of different transcription factor is specific. DNA binding domain has several families according to its structure character, such as helix-turn-helix, zinc finger, winged helix and so on [44-47].

The structure motif(s) of transcription factor fulfill transcriptional activation is called transcriptional activation domain (TAD). By analyzing the amino acid sequence, TAD has been classified based on the abundance of particular amino acid [48, 49]. In this way transcription factor are divided into glutamine rich, proline rich, acidic and so on. Recently, with the assistant of computational biology, a nine-amino-acid transcriptional activation domain (9aaTAD) defines a novel domain classification of the eukaryotic and yeast transcription factor family [50]. Most researches showed that the transcriptional activation process accompanied by the conformational changes of both TAD and its binding sites, although the initiator of the changes may not be the same side all the time [51-54].

Transcription factors are important in cell cycle control, development and intercellular signaling transduction. Some human diseases have been the results of transcription factors' mutations. Many transcription factors were found to be

important for tumor related signaling and were considered to be tumor suppressors or oncogenes. For example, over 50 percent of tumors contain a point-mutation or deletion mutation of the p53 gene in human species [55-57]. Several transcription factors, such as hypoxia inducible factor (HIF), Sp1 and so on, are considered to be important for tumor angiogenesis process [58-63]. Some of them were found to be angiogenic factor of different types of tumors including pancreatic cancer.

## ANGIOGENESIS AND PANCREATIC CANCER PATHOGENESIS

Capillaries are the lifeline of all live tissue including and especially for tumor. Oxygen and nutritious delivery and waste removal are the basic physiological function of blood circle system fulfilled ultimately by capillaries. Additionally, tumor needs to be near capillaries to metastasize to distance, which leads to its fatality property. Due to the high proliferation rate of tumor cells, tumor keeps on expansion and invading the around normal tissue. This process totally relies on the growth of new blood vessels. The physiological process of the new blood vessels growth from the existing vessel is called angiogenesis [64-66]. There are two types of angiogenesis. One is sprouting angiogenesis and the other is intussusceptive angiogenesis. Sprouting angiogenesis enables entire new vessels to grow from existing blood vessel and occurs at a rate of several millimeters per day. While intussusceptive angiogenesis actually form new blood vessel through splitting the existing blood vessel and is also called splitting angiogenesis [67]. But it is important because it allows a vast increase in the



number of capillaries with relative limited number of endothelial cells. When there are not enough resources especially during the embryonic development, it is a very important way to develop new vessels.

The concept of angiogenesis appears in early 1970's. It was recognized during the research of neoplastic as well as non-neoplastic diseases such as psoriasis, atherosclerosis, rheumatoid arthritis, infantile hemangiomas and so on [68-73]. Angiogenesis occurs in not only the pathological process but also the normal physiological processes including reproduction, development and wound healing. The following discussion will focus on tumor related pathological process. Pathological angiogenesis may lead to bleeding, vascular leakage and tissue destruction.

Angiogenesis is a process orchestrated by a lot of angiogenic factors as well as angiogenic inhibitors. With the progress of angiogenesis research, lots of natural angiogenic inhibitors were found and the concept of anti-angiogenic therapy also sprouted and grew rapidly especially at the mid 1990's [74-81]. Considering the fact that angiogenesis is important to tumor growth and metastasis, anti-angiogenic therapy may be one potential selection of pancreatic cancer patients. It may produce enhanced efficacy and long-term survival [66, 82, 83]. The acquisition of angiogenic phenotype of tumor tissue and its following maintenance are mainly through over-expression of angiogenic molecules. The over-expression of the molecules is activated by growth factors, stress factors and cytokines secreted by tumor cells. Transcription factors between the

upstream signal factors and downstream angiogenic factors are the neck part of the regulatory pathway of tumor angiogenesis and maybe a good target [58].

To achieve effective treatment modalities of pancreatic cancer, new targets need to be identified. It is crucial to fully understand the cellular and molecular mechanisms of the development and progression of tumor angiogenesis. Lots of the reported angiogenic and anti-angiogenic factors released by tumor and host cells are the potential targets [58, 84, 85]. Angiogenesis process is regulated by the interaction of these factors and then regulates the survival and metastasis of pancreatic tumor [58, 86, 87]. Among the numerous angiogenic factors discovered till now, studies have identified vascular endothelial growth factor as a key mediator of tumor angiogenesis in most tumor types [88-90]. Other identified angiogenic factors include platelet-derived growth factor (PDGF) and PDGF receptor [91, 92], fibroblast growth factors (FGF) and their receptors [93, 94], epidermal growth factor (EGF) family and their receptors [84, 95], insulin-like growth factor-I (IGF-I) and IGF-I receptor [96, 97], and hepatocyte growth factor (HGF) and the receptors, such as Met [98, 99].

## HYPOTHESIS AND SPECIFIC AIMS

Several laboratories including ours reported that transcription factor Sp1 is an important angiogenic molecule in several tumor types [61, 100-104]. Sp1 is a zinc finger protein, and it is essential to the trans-activation of many genes that contain GC boxes or GT boxes (also called Sp1 site) in their promoters. Sp1 has been considered to be a basal transcription factor because of its constitutive

expression; however increasing evidence suggests that several of biological functions are regulated by Sp1, such as cell growth, survival, and differentiation. These biological functions are closely related to tumor development and progression [105, 106]. It has been reported by our lab that Sp1 over-expression is correlated with the angiogenesis and poor prognosis of human gastric and pancreatic cancer [60, 61, 107-109]. With these experimental evidences, I hypothesize that **transcription factor Sp1 is an effective target of anti-angiogenic therapy in human pancreatic cancer**. To test this hypothesis, I propose the following specific aims:

1. **Determine the definitive role of Sp1 in pancreatic cancer angiogenesis.** To fulfill this aim, first whether Sp1 expression is correlated with angiogenic phenotype in human pancreatic cancer patient will be determined by state-to-art immunohistochemistry technique and corresponding statistics. Second, Sp1 function in pancreatic cancer angiogenesis will be defined with both molecular biology and cellular biology technology.

2. **Design and test anti-Sp1 targeting therapy effect and Sp1 function in the up-to-date anti-VEGF targeting therapy in human pancreatic cancer.** First, Sp1 inhibitor mithramycin function will be defined to determine whether Sp1 manipulations will improve those current regimens of pancreatic cancer therapies in mouse model. Second is to determine VEGF neutralization monoclonal antibody Bevacizumab therapy effect and optimize mithramycin and

Bevacizumab combination treatment in mouse model. Third, the underlying mechanism of the anti-angiogenic therapy will be explored.

**3. Determine therapeutic effect of combined transcriptional repression and protein degradation of Sp1 for treatment of pancreatic cancer in mouse models.** First is to determine the ability of mithramycin and/or tolfenamic acid to manipulate Sp1 *in vitro*. Second, therapeutic effect of mithramycin and/or tolfenamic acid will be determined on pancreatic cancer with mouse model. Third, the corresponding molecular mechanism of the anti-Sp1 anti-angiogenesis therapy will be explored.

In summary, this study will be to determine the definitive role of transcription factor Sp1 in human pancreatic cancer and to design and test anti-Sp1 targeting therapy in mouse models. The long-term goal of the study would be to translate the therapeutic designs to clinic and benefit pancreatic cancer patients.

# CHAPTER I

## Definitive Role of Sp1 in Pancreatic Cancer Angiogenesis

### INTRODUCTION

#### PANCREATIC CANCER ANGIOGENESIS

Just like in most solid tumors, angiogenesis plays important roles in both pancreatic tumor growth and metastasis. A range of angiogenic factors regulates angiogenesis process [82, 88, 90]. VEGF, PDGF and bFGF were considered to be the most important molecules among the various of angiogenic factors [82]. In pancreatic cancer, VEGF family was the most important angiogenic factor and has been well documented [88-90]. VEGF family has 6 members (Figure1), VEGF or VEGF-A (located on 6p12-p21) [110], VEGF-B (located on 11q13) [111], VEGF-C (located on 4q34) [112], VEGF-D (located on Xp22.31) [113], VEGF-E (found in virus ) [114], and placenta growth factor (PlGF, located on 14q24-q31) that is 53% identity to the platelet-derived growth factor-like region of VEGF-A [115]. VEGF-A is the most well-characterized member and has at least 6 isoforms. The gene coding region spans 14kb and has 8 exons (exon 6 and 7 are the membrane binding domain) [116, 117]. VEGF<sub>189</sub> and VEGF<sub>206</sub> anchor in the membrane and stay in the extracellular matrix. VEGF<sub>165</sub> (without exon 6) can be secreted or membrane bonded. VEGF<sub>121</sub> (without exon 6 and 7) is a free

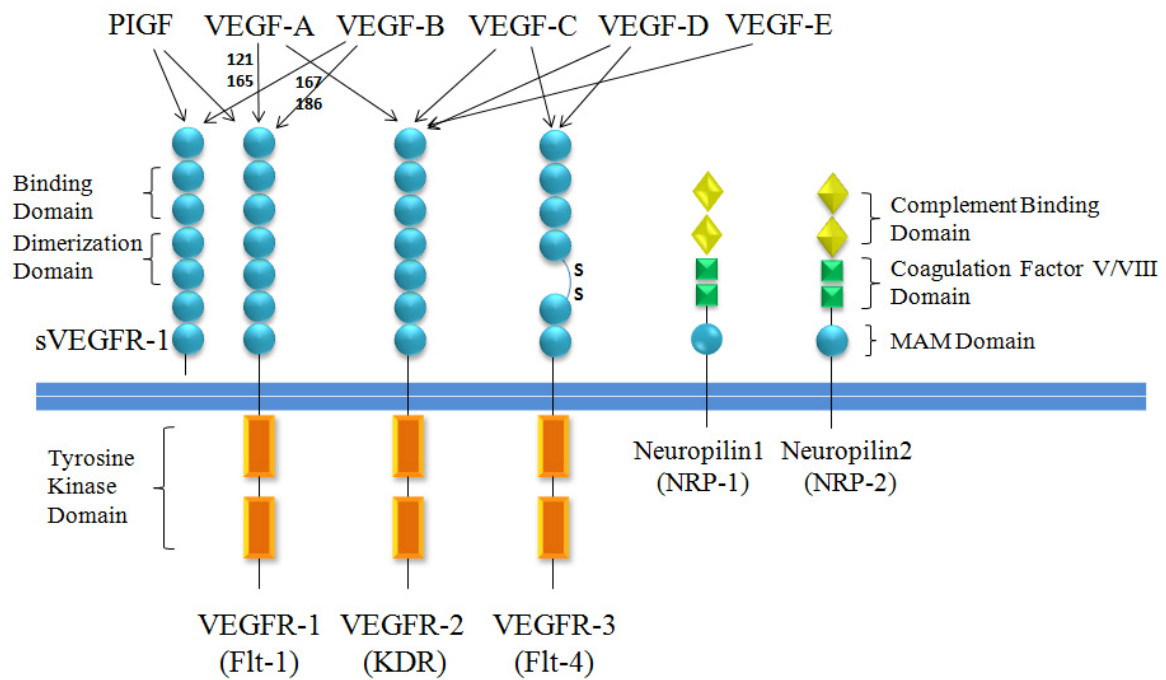
diffusible protein. VEGF<sub>121</sub> and VEGF<sub>165</sub> bind to and activate VEGFR-2 to induce mitogenic and permeability-enhancing. Others only work on permeability-enhancing. VEGF-B has 2 isoforms, VEGF<sub>167</sub> and VEGF<sub>186</sub>[118]. They can all form heterodimer with VEGF-A. VEGF-C and D are more selective growth factors for endothelium and lymphatic vessels by binding VEGFR-3.

VEGF family members release their signal by binding VEGF receptor in the cell surface [119]. There are 5 VEGF receptors (Figure1), 2 of them were identified from endothelial cells first. They are the 180kDa fms-like tyrosine kinase 1 (Flt1 or VEGFR1) and the 200kDa kinase insert domain receptor (KDR or VEGFR2). VEGFR2 binds VEGF-A, VEGF-C, VEGF-D, VEGF-E, whereas VEGFR1 binds VEGF-A, VEGF-B and PlGF. They are expressed mainly on vascular cell of endothelial origin. The third receptor is the 180kDa Flt4 (or VEGFR3). It binds VEGF-C and VEGF-D. Similar to VEGFR2, VEGFR3 is mainly expressed on endothelial cell during early development of embryo [120, 121]. However its expression is mostly limited to lymphatic endothelial cell in the differentiated tissues [122]. The other two receptors were the 130-140kDa isoforms Neuropilin-1 (brief as NRP-1), Neuropilin-2 (brief as NRP-2). NRP-1 can bind VEGF<sub>165</sub>, and PlGF. NRP-2 is similar to NRP-1 and can bind VEGF<sub>165</sub>, VEGF<sub>145</sub>, PlGF and VEGF-C. NRP-1 and 2 are different from other family members [123]. They have a short intracellular domain. The short intracellular domain cannot transduce biological signals independently. It was considered that they may act as a co-receptor for VEGFR-1 and VEGFR-2. The interaction of ligand and receptor

could be enhanced and the signal might be amplified by their anticipation in the complex [123].

Studies have shown that anti-angiogenesis therapy inhibits pancreatic tumor growth in mouse model. Currently targeting VEGF signaling and function is still a hot spot in anti-angiogenesis, tumor growth and metastasis. Strategies have been developed, including the use of anti-VEGF antibodies, targeting VEGF receptors to directly interfere the signal effect [124, 125]. Additionally, multiple genes, identified in pancreatic cancer switch of the "angiogenic network" [126, 127], were also proposed to be the anti-angiogenic targets and targeted in experimental mouse model [58, 128]. These reported results from different experimental and clinical processes with different approaches showed the importance of the angiogenic process in pancreatic cancer. Also, replication deficient recombinant adenovirus mediated vasostatin was evaluated in human pancreatic cancer mouse model and shown to be efficient gene therapy for pancreatic carcinoma [129].

How the angiogenic molecules regulate angiogenesis process in pancreatic cancer is still not clear, while it is crucial to understanding the mechanism for designing effective anti-angiogenic therapies. Several reports including ours indicated that Sp1 plays a key role in VEGF and angiogenesis regulation of human pancreatic cancer [61, 130].



**Figure 1 VEGF family members and their receptors** VEGF family has 6 members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E (Virus protein, not shown here) and PIGF. VEGF has 5 receptors, VEGFR-1, VEGFR-2, VEGFR-3, Neuropilin-1 and Neuropilin-2.



## **SPECIFICITY PROTEIN 1 (Sp1) AND PANCREATIC CANCER**

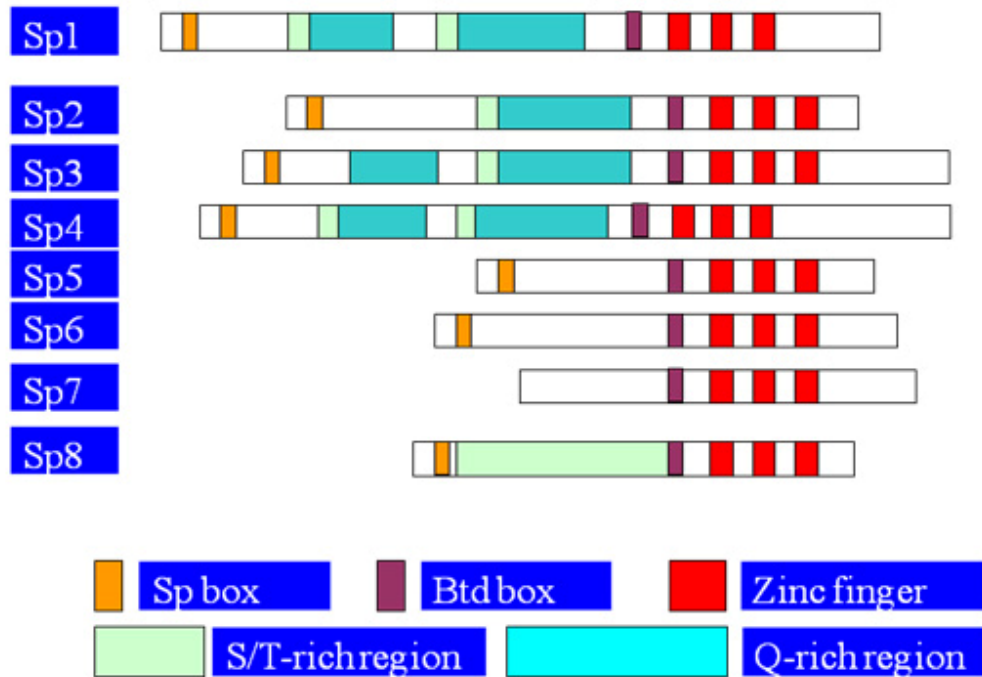
Sp1 is a transcription factor that has three tandem zinc finger domains in its carboxyl terminal. It was the first sequence-specific regulator of mammalian gene transcription and was cloned by James Kadonaga in 1987 [131]. It can bind to Sp1 sites in the early promoter of SV40 virus with the carboxyl terminal characteristic tandem Cys<sub>2</sub>His<sub>2</sub> ((Tyr, Phe)-Xaa-Cys-Xaa<sub>2,4</sub>-Cys-Xaa<sub>3</sub>-Phe-Xaa<sub>5</sub>-Leu-Xaa<sub>2</sub>-His-Xaa<sub>3-5</sub>-His) zinc finger structure. It is believed that Sp1 can bind to GC and GT boxes (Sp1 site) in promoter and regulates the transcription. Sp1 sites were found in numerous mammalian gene promoters. Sp1 was thought to be responsible for recruitment of TATA-binding protein (TBP) and to fix the transcription starting site and was considered as a basal transcription factor.

Researchers have cloned transcription factors similar to Sp1 named as Sp2, Sp3, Sp4, Sp5, Sp6, Sp7 and Sp8 [132]. These eight molecules form the Sp multi-gen family. They all have the characteristic three tandem zinc finger structure at carboxyl terminal (Figure 2). Sp2, Sp3, and Sp4 also have glutamine-rich trans-activation domain. But Sp2 is mainly expressed in neural system. Sp3 and Sp4 are considered to be closely related to Sp1. The tandem zinc finger structure character is also appeared in Krüppel-like factor family. These transcription factors may have the transcriptional activation or repression function.

Sp1 has been considered to be a basal factor since it was discovered. But more and more experimental evidence indicated that it can regulate a variety of

biologic functions. In cancer biology, these functions are important to tumor development and progression [60, 108, 109, 132-134]. Recently, several studies have reported that other Sp family members are also over-expressed in pancreatic cancer cells. The elevated expression of these molecules was also found in tumors compared with non-tumor tissue. These alterations play important role in the pancreatic cancer malignance nature.

Pancreatic cancer is believed to be resulting from the genetic alternation of lots of genes. The average of the alternation was reported to be 63 [135]. All these alternations finally converge their signaling to a small number of transcription factors. Sp1 is considered to be one of the several oncogenic transcription factors and is important in pancreatic cancer pathogenesis [58, 132].



**Figure 2 Sp family members and their structures** The Sp box (SPLALLAATCSR/KI) at the N-terminus is highly conserved of the Sp proteins. It contains an endoproteolytic cleavage site that is close to a proteasome-dependent degradation target region of Sp1 *in vitro*. The (Btd) Buttonhead element is important in the synergistic activation by Sp protein with sterol-regulatory element-binding proteins. The carboxyl-terminal has the family marker region, featuring three Cys2His2 zinc “fingers”, which are required for sequence-specific DNA binding to GC-rich promoter elements. The (S/T) serine/threonine-rich subregions located next to the glutamine-rich regions are believed to be involved in post-translational modification. The (Q-rich) glutamine-rich portions of A and B are required for trans-activation. Each of which can stimulate transcription when tethered to DNA through a DNA-binding domain.

## **MATERIAL AND METHODS**

### **Information of Human Tissue Specimens and Patient**

Specimens of human pancreatic cancer tissue from the Pancreatic Cancer Tissue Bank (Shanghai Jiaotong University-Affiliated Ruijin Hospital) were used. These specimens were well archived. Pancreatic tumors in these patients were all primary pancreas tumors. Patients were diagnosed and treated at Ruijin hospital from 2002 to 2004. All of the patients did not received pre-operative chemotherapy or radiation therapy before surgery. 35 patients were selected to represent all stages of pancreatic tumor. The group of studied patients consisted of 20 men (57.1%) and 15 women (42.9%). 16 patients (45.7%) were aged > 60 years, and 19 patients (54.3%) were aged < 60 years (mean age, 60.4 years). The tumor, lymph node, metastasis (TNM) criteria was used to staging the patients. There are 4 patients (11.4%) had stage I disease, 26 patients (74.3%) had stage II disease, 3 patients (8.6%) had stage III disease, and 2 patients (5.7%) had stage IV disease.

### **Human Normal and Tumor Tissue Specimens Immunohistochemical Staining**

Sections (5  $\mu$ m thick) of formalin-fixed, paraffin-embedded pancreatic tumor specimens were prepared and processed for immunohistochemistry to detect Sp1 and VEGF protein expression and tumor MVD by using anti-Sp1, anti-VEGF,

and anti-CD34 antibodies respectively. Antigen retrieval was performed with 0.05% saponin for 30 min at room temperature. Endogenous peroxidase was blocked using 3% hydrogen peroxide in PBS for 12 min. The specimens were incubated for 20 min at room temperature with a protein blocking solution consisting of PBS (pH 7.5) containing 5% normal donkey serum and bovine serum albumin and then incubated at 4°C in polyclonal antibody against human Sp1 (clone PEP2) or VEGF overnight. The samples were then rinsed and incubated for 1 hour at room temperature with peroxidase conjugated anti-rabbit IgG. Next, the slides were rinsed with PBS and incubated for 5 min with diaminobenzidine. The sections were washed three times with distilled water, counterstained with Mayer's hematoxylin (Richard Allan Scientific, Kalamazoo, MI), and washed once with distilled water and PBS. Afterward, the slides were mounted using Universal Mount (Biomedica Corporation, Foster City, CA) and examined using a bright-field Leica microscope.

A positive reaction was indicated by a reddish-brown precipitate. Depending on the percentage of positive cells and staining intensity, staining was classified into three groups: negative, weak positive and strong positive. Specifically, the percentage of positive cells was divided into five grades (percentage scores) : <10% (1), 10–25% (2), 25–50% (3), 50–75% (4), and >75% (5). The intensity of staining was divided into four grades (intensity scores): no staining (1), light brown (2), brown(3), and dark brown (4). Sp1 and VEGF staining positivity were determined by the formula: overall scores = percentage score x intensity score.

The tissues sections were scored by 2 independent investigators prior knowing the patient outcomes, and the mean values of 2 independent scores are presented [60, 130, 136].

### **Cell Lines Information and the Corresponding Culture Conditions**

The human pancreatic adenocarcinoma cell lines AsPC-1, BxPC-3, and PANC-1 were purchased from the American Type Culture Collection (Manassas, VA). FG human pancreatic adenocarcinoma cells were established by Vezeridis et al [137]. All of the cell lines were maintained in plastic flasks as adherent monolayer in minimum essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and a vitamin solution (Flow Laboratories, Rockville, MD). Human umbilical vein endothelial cells (HUVECs) were purchased from BD Biosciences (San Jose, CA) and were cultured with the endothelial cell culture medium (BD Biosciences, San Jose, CA) according to the manufacturer's instructions.

### **Small-interfering RNA Information**

Small interfering RNA (siRNA) reagents, including transfection reagents, were obtained from Invitrogen (Carlsbad, CA). A Sp1 Stealth RNAi duplex oligo was used to knock down Sp1 expression in pancreatic cancer cells. Non-targeting scrambled Stealth RNAi Negative Control siRNA were used as negative controls

according to the manufacturer's instructions. *In vitro* transfection was performed by using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA).

### **Animals Information and Animal Care**

Female athymic nude mice and C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). The 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 facilities 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 in M D Anderson Cancer Center animal facility.

### **Animal Models of Tumor Growth and Metastasis**

Tumor cells ( $1 \times 10^6$  cells per mouse) were injected into the subcutis or pancreas of nude mice in groups of 5 to 10 mice. The animals were killed 60 days after the tumor-cell injection or when they had become moribund. Next, their primary pancreatic tumors were harvested and weighed. In addition, the liver from each mouse was removed and fixed in Bouin solution (BBC Biochemical, Dallas, TX) for 24 hours to differentiate the neoplastic lesions from the organ parenchyma; metastases on the surface of the liver were counted under a dissecting microscope.

## **Western Blot Protocol**

Whole-cell lysates of human pancreatic cancer cell lines or tissues were prepared [61]. Standard Western blot analyses were performed by using corresponding antibodies and the anti-rabbit immunoglobulin G (IgG) antibody, a horseradish peroxidase–linked F(ab')<sub>2</sub> fragment obtained from donkey (Amersham, Arlington Heights, IL) as probing molecule. Equal protein-sample loading was monitored by probing the same membrane filters with an anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody [61]. The probe proteins were detected by using the Amersham enhanced chemi-luminescence system according to the manufacturer's instructions.

## **Immunohistochemical Staining of Human Pancreatic Tumor Xenograft Specimens**

For VEGF and Sp1 staining of human pancreatic xenograft specimens, sections (5 µm thick) from formalin-fixed, paraffin-embedded pancreatic tumor specimen were de-paraffinized in xylene and rehydrated in graded alcohol. For CD31 staining, frozen sections (6 µm thick) of tumor specimens were fixed with acetone. Sp1 and VEGF expression and MVD status in the sections were assessed as described previously.

## **Matrigel Plug Assay**



Matrigel (200  $\mu$ l) containing  $2 \times 10^6$  cells of different treated pancreatic cancer cells described above was injected subcutaneously into nude mice (2 injection sites per mouse). The Matrigel plugs were recovered from the mice 10 days after injection and carefully stripped of host tissues [138-142]. After photography, the Matrigel plugs were weighed and homogenized in 1 ml of distilled water and then centrifuged at 10,000 revolutions per minute for 5 minutes. The supernatants were collected for hemoglobin measurement using Drabkin solution (Sigma-Aldrich, Saint Louis, CA) and measured with a Spectra Max M5 Microplate Manager enzyme-linked immunosorbent assay reader at 540 nm according to the manufacturer's instructions. The relative hemoglobin concentrations were calculated and further normalized according to the weights of the plugs.

### **Endothelial Cell Tube Formation Assay**

A standard tube formation assay was performed using human umbilical vein endothelial cells (HUVECs) [138, 141]. PANC-1 cells ( $3 \times 10^6$ ) of different treatments were cultured in 1 ml of serum-free medium for 24 hours, and the medium was collected and centrifuged to remove any cell debris before its use as a conditional medium. HUVECs were cultured with endothelial cell culture medium (BD Biosciences, San Jose, CA). 24-well plates were coated with reconstituted Matrigel (BD Biosciences, San Jose, CA) following the manufacturer's directions.  $2 \times 10^4$  HUVECs were transferred to each well of 24-well plates and incubated in culture medium at 37°C, 5% CO<sub>2</sub> for adhesion. Then

culture medium was replaced with the conditional medium harvested from different treated cells and incubated for 18 hours [143]. The degree of tube formation was assessed as the percentage of cell surface area versus total surface area. Pictures were captured with bright-field Leica microscopy using a Spot-RT digital camera equipped with a Spot Imaging 4.7 program.

### **Statistical Analysis**

For studies using human specimens, the chi-square test was performed to determine the significance of the differences between the covariates (Sp1, VEGF, and MVD). For *in vitro* and *in vivo* studies, each experiment was performed independently at least twice with similar results; 1 representative experiment is presented. The significance of the *in vitro* data was determined using the Student *t* test (two-tailed), whereas the significance of the *in vivo* data was determined by using the Mann-Whitney *U* test.  $P < 0.05$  was deemed significant.

## RESULTS

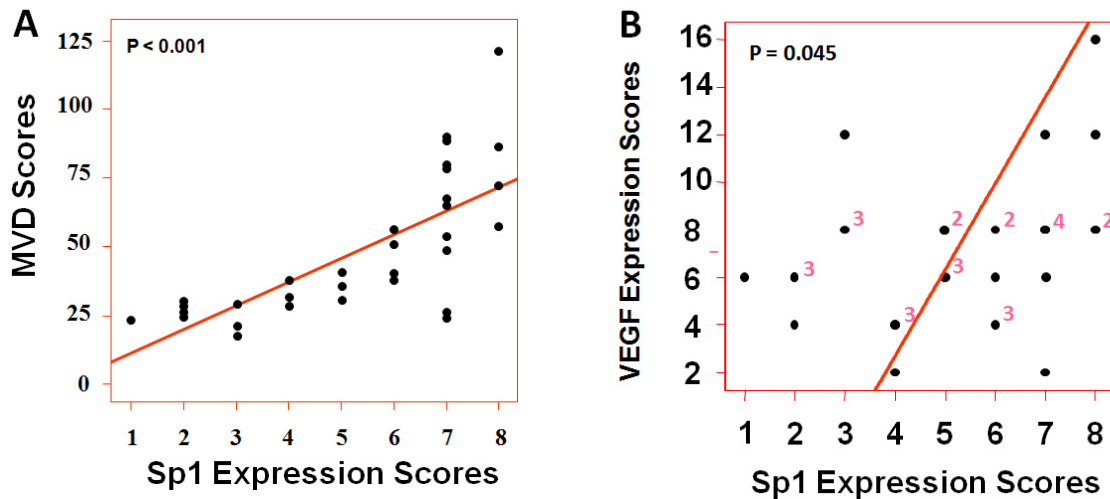
### **Correlation of Sp1 expression levels with VEGF expression and MVD in human pancreatic cancer patients specimen**

Researchers have reported the importance of Sp1 in pancreatic cancer and that the expression of VEGF, one of the most key angiogenic molecules, depends on Sp1, which have suggested the role of angiogenesis in pancreatic cancer. But studies have not yet investigated the relation between MVD status, one of the angiogenic index, VEGF expression and Sp1 expression. The underlying interaction between these 3 factors in pancreatic cancer is not clear.

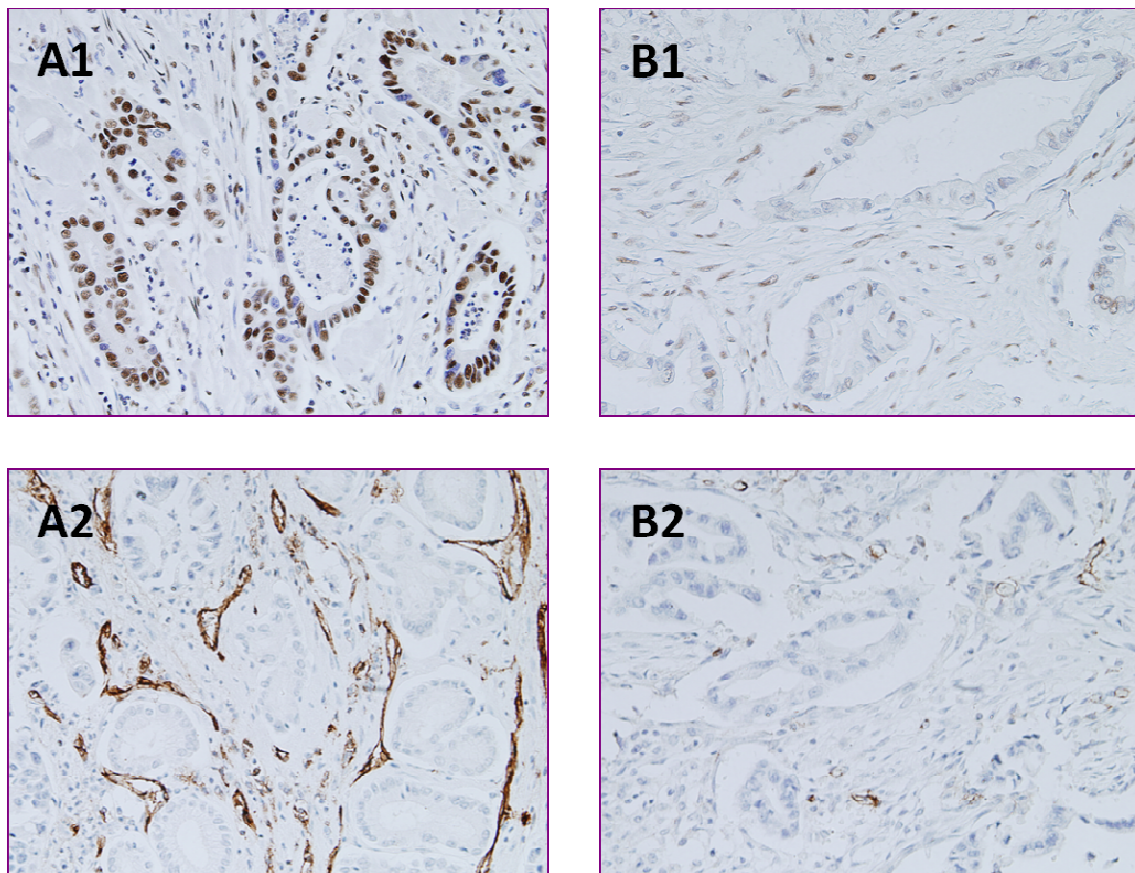
To answer this important question, 35 primary pancreatic cancer tissue specimens were used to evaluate Sp1, VEGF expression and MVD status using immunohistochemistry staining and corresponding statistics. It was found that the MVD status was correlated highly with Sp1 expression ( $P < 0.001$ ) (Fig. 3A1). The level of Sp1 expression was correlated significantly with VEGF expression level ( $P < 0.05$ ) (Fig. 3A2).

These co-relationships were confirmed by analyzing the consecutive sections of human pancreatic cancer specimens. The representative immunohistochemistry staining pictures of two representative patients' specimens (selected from those 35 patients) were shown in Figure 4. The patient that has strong Sp1 expression (Fig. 4A1) has high MVD (Fig. 4A2), while the patient that has very weak Sp1 expression (Fig. 4B1) has low MVD and very small micro vessel (Fig. 4B2).

Obviously the pattern of Sp1 expression was consistent with MVD status. Therefore, it can be concluded that Sp1 can regulate human pancreatic cancer angiogenesis with the supportiveness of these clinical evidence shown in Figure 3 and Figure 4.



**Figure 3 the level of transcription factor Sp1 expression correlates with the microvessel density (MVD) status in human pancreatic cancer.** (A) Tissue sections were prepared from formalin-fixed, paraffin-embedded pancreatic tumors (35 patients). Immunohistochemical staining was performed by using specific antibodies against Sp1, vascular endothelial growth factor (VEGF), and CD34 (MVD) and was scored as described in Material and Methods. The direct correlations between Sp1 expression and MVD status (A1) ( $P < 0.001$ ) and between Sp1 expression and VEGF expression (A2) ( $P < 0.05$ ) were analyzed using the Person chi-square test.



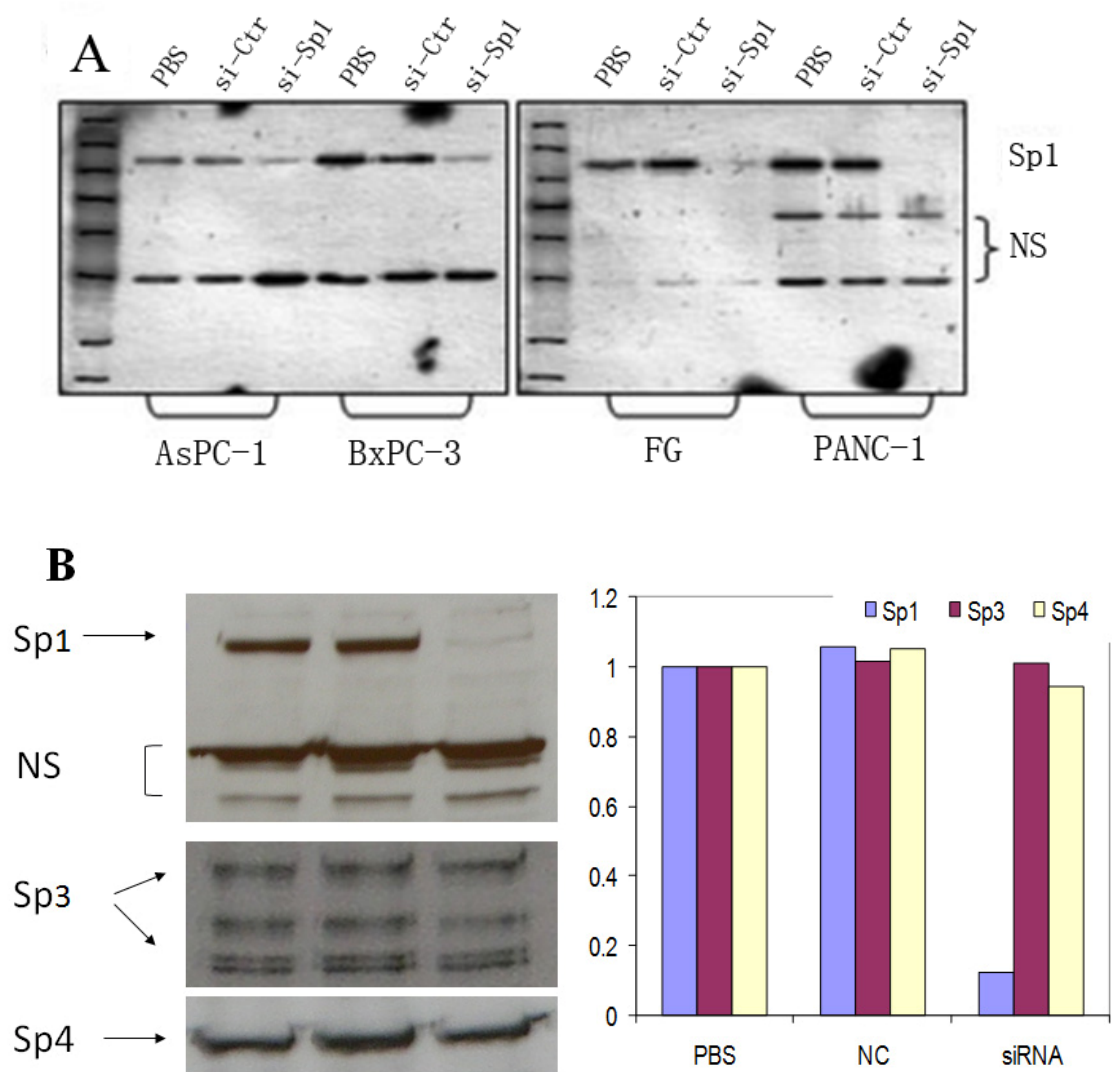
**Figure 4 Representative immunohistochemistry staining results.** Two sets of consecutive tissue sections that were positive (A1) and negative (B1) for Sp1 expression also were stained for CD34 expression (A2, B2). Representative photomicrographs are shown (original magnification,  $\times 100$ ). It is noteworthy that Sp1 expression correlated directly with MVD status.

## **Sp1 expression were specifically knockdown in human pancreatic cancer cells**

Several pancreatic cancer cell lines, including AsPC-1, BxPC3, FG and PANC-1 were transfected with Sp1 small-interfering RNA (si-Sp1) or control siRNA duplexes (si-Ctr) using Lipofectamine 2000 according the direction from Invitrogen (Carlsbad, CA). Cells were harvested after 24 hours of treatment to prepare whole cell lysates. Western blot was used to analyze protein level changes.

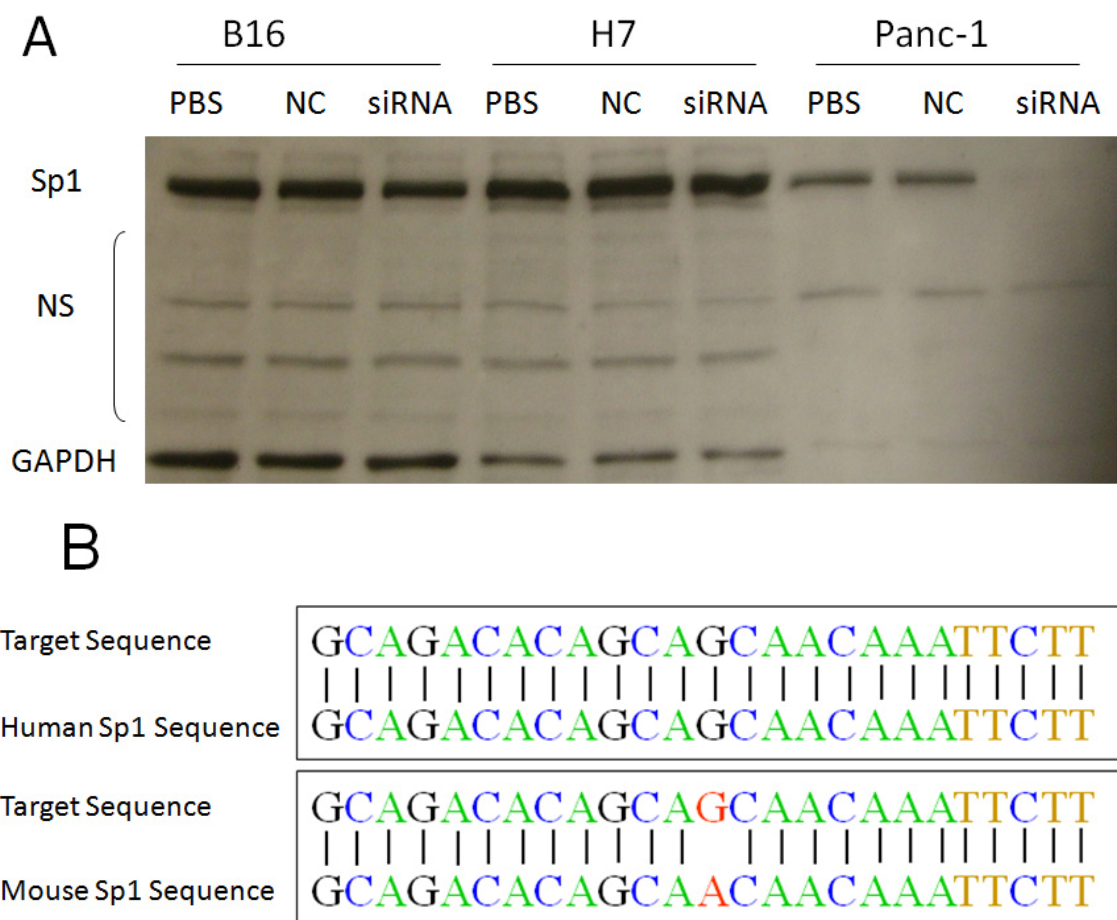
Sp1 expression down-regulation was confirmed in these cells (Fig. 5A). Sp1 protein was knockdown to less than 10% and even near 0% in particular cell line like PANC-1. In Sp1 protein family, Sp3 and Sp4 are close to Sp1. The specificity of Sp1 knockdown but not Sp3 or Sp4 was confirmed with Western Blot and the quantitative result was shown (Fig. 5B).

The specificity of Sp1 knockdown in different species was also determined with tumor cell lines from human and mouse. Mouse melanoma cell line B16 and pancreatic tumor cell line H7 were selected. The specificity was confirmed with Western Blot (Fig. 6A). The siRNA targeting sequence is also listed and there is only one nuclear acid unmatched with mouse sequence (Fig. 6B). But the mouse Sp1 protein level has no change in comparison with human Sp1.



**Figure 5 Knockdown of transcription factor Sp1 expression.** For Sp1 knockdown, AsPC-1, BxPC-3, FG, and PANC-1 cells were transfected with Sp1 small-interfering RNA (si-Sp1) or with control small-interfering RNA (si-Ctr). Sp1 expression in these cells was confirmed by using Western blot analysis (A). PBS indicates phosphate-buffered saline; NS, nonspecific bands. The specificity of Sp1 knockdown in Sp protein of these cells was confirmed by using Western blot analysis and was quantitated (B).





**Figure 6 Specificity of knockdown of transcription factor Sp1 expression.** Human pancreatic cell line PANC-1, mouse melanoma cell line B16 and pancreatic cell line H7 were transfected with Sp1 small-interfering RNA (si-Sp1) or with control small-interfering RNA (si-Ctr). Sp1 expression in these cells was confirmed by using Western blot analysis (A). PBS indicates phosphate-buffered saline; NS, nonspecific bands. The siRNA target sequence and the corresponding regions of human and mouse were shown (B). The only unmatched nuclear acid was marked in red.

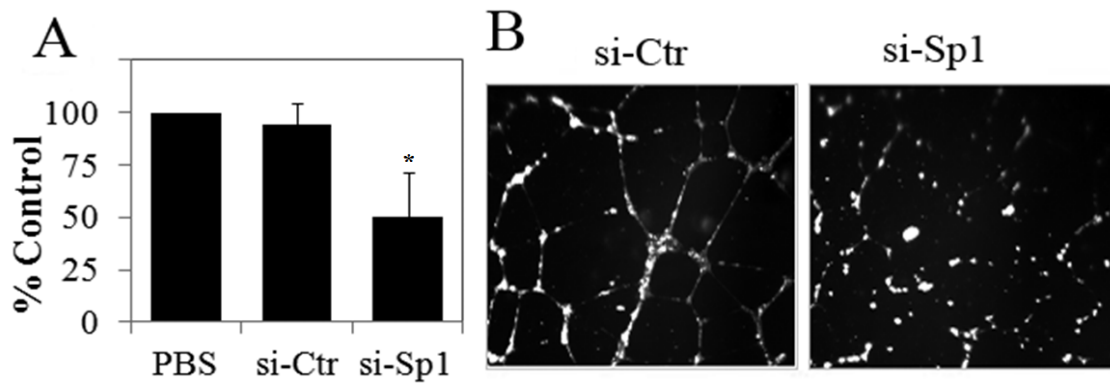
## **Human pancreatic cancer cells lost the angiogenic potential after Sp1 expression were knockdown**

To determine the Sp1 function in pancreatic cancer angiogenesis, tube-formation and Matrigel plug assay were performed as described in Material and Methods.

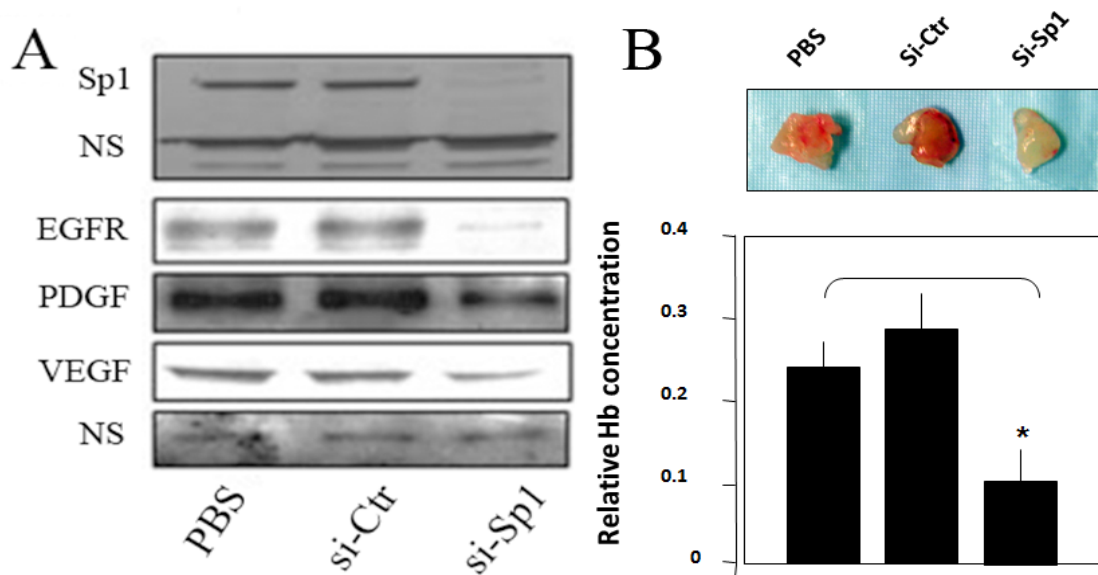
Briefly, PANC-1 cells were transfected with control, si-Ctr or si-Sp1. The angiogenic potential of the supernatants of PANC-1 cells was determined using an endothelial cell tube formation assay following the protocol provided by supplier. The degree of tube formation was assessed as the percentage of cell surface area versus the total surface area and the statistic result was shown (Fig. 7A). Representative photomicrographs of different treatment groups were taken *in situ* for tube formation of HUVECs incubated in the supernatants of different treatment groups (Fig. 7B). As it shown that PANC-1 cells transfected with si-Sp1 reduced the capacity of its supernatant's stimulate tube formation of endothelial cells when comparing with the capacity of supernatants of PANC-1 cells transfected with si-Ctr.

The expression of several genes was analyzed after Sp1 knockdown with Western Blot (Fig. 8A). As it revealed that the expression of EGFR, PDGF, and VEGF were down regulated. These are consistent with other researchers' reports. Sp1 sites in the promoter region of these angiogenic growth factors are the key regions to regulate their mRNA synthesis.

To further determine Sp1 knockdown effect on pancreatic cancer cell angiogenesis, Matrigel plug assay was performed according to Material and Methods. The angiogenic potential of pancreatic cancer cells was impaired *in vivo* (Fig. 8B). The data suggested that the knockdown Sp1 protein expression impaired the angiogenic potential of pancreatic cancer cells.



**Figure 7 Human pancreatic cancer cells lost the angiogenic potential after knockdown of transcription factor Sp1 expression *in vitro*.** The angiogenic potential of the supernatants of PANC-1 cells, PANC-1 cells transfected with si-Ctr, and PANC-1 cells transfected with si-Sp1 was determined by using an endothelial cell tube formation assay (A). Representative photomicrographs were taken *in situ* of human umbilical vein endothelial cell tube formations in the supernatants of PANC-1 cells treated with si-Ctr or si-Sp1 (B). \*  $P < 0.05$  in a comparison between the treated and respective control groups.



**Figure 8 Knockdown of transcription factor Sp1 expression attenuates human pancreatic cancer cell angiogenic potential *in vivo* and gene expression analyses *in vitro*.** PANC-1 cells were treated as described above, and total protein lysates were prepared. Western blot was used to analyze vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), and platelet-derived growth factor (PDGF) protein by using corresponding specific antibodies (A). For Matrigel plug assay, Matrigel (500  $\mu$ L) that contained  $2 \times 10^6$  PANC-1 cells or PANC-1 cells treated with si-Ctr or si-Sp1 was used as described in Materials and Methods (B) (Hb indicates hemoglobin). It is noteworthy that the down-regulation of Sp1 expression impaired the angiogenic potential of pancreatic cancer cells *in vitro* and *in vivo*. \*  $P < 0.05$  in a comparison between the treated and respective control groups.

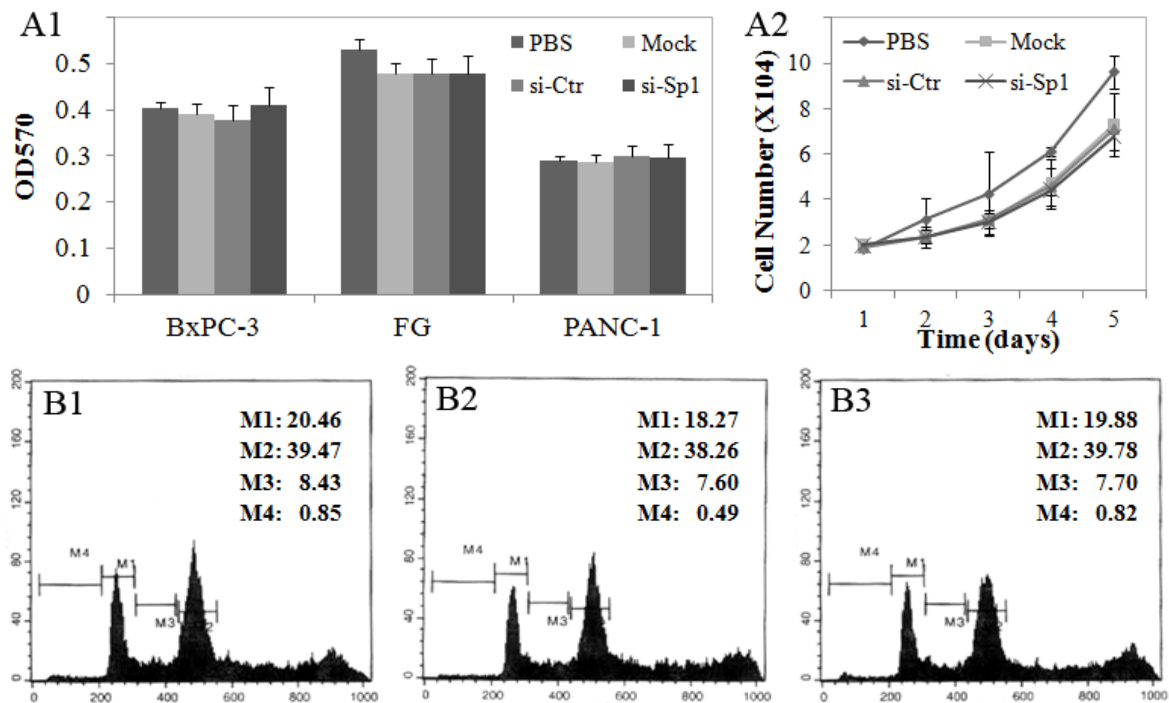
## **Knockdown of Sp1 expression suppressed human pancreatic cancer cells growth *in vivo* and correlated with impaired angiogenesis**

To determine whether cell cycle was affected after knockdown Sp1 expression *in vitro*, MTT assay and FACs analysis were performed as described in Material and Methods.

Briefly, BxPC-3, FG, and PANC-1 cells were incubated in medium alone or in medium that contained Lipofectamine with si-Ctr or si-Sp1 for 3 days. 3-(4, 5-dimethylthiazol-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to assess the cell growth (Fig. 9A1). The viable cells that can be stained by trypan blue were counted (Fig. 9A2). The level of cell cycle progression was determined by using fluorescence-activated cell sorter analysis with propidium iodide staining (Fig. 9B). It was observed that the difference of S + G2 + M phase cells was about 2 percent. Knockdown Sp1 protein expression had minimal effect on tumor cell growth *in vitro*.

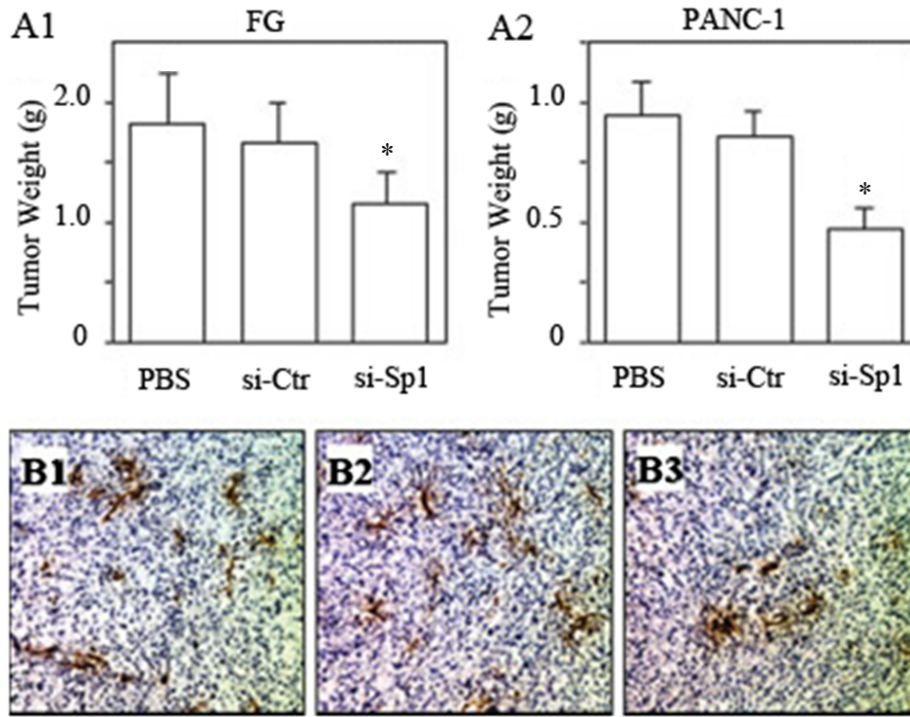
Whether knockdown of Sp1 expression affects tumor growth *in vivo* was then determined. FG or PANC-1 cells transfected with si-Ctr or si-Sp1 were injected into the pancreas of mice. Mock transfected cells were also used as control. 10 mice were used for each treatment group. Mice were sacrificed at the end of second month after the injection. Tumors were harvested and weighted for extended analysis.

When assessed by tumor weight, it was found that FG or PANC-1 cells transfected with si-Ctr produced larger tumors. But the cells transfected with si-Sp1 produced smaller tumors (Fig. 10A). The MVD status of xenograft tumor tissue specimens was processed and photomicrographed as described in Material and Methods. Representative pictures of each treatment group from PANC-1 xenograft tumor were shown (Fig. 10B). It is clearly showed that, PANC-1 cells transfected with si-Sp1 formed tumors had less vascular than those formed by cells transfected with si-Ctr or mock transfection.



**Figure 9 Knockdown of transcription factor Sp1 expression does not affect the *in vitro* growth of human pancreatic cancer cells.** (A) BxPC-3, FG, and PANC-1 cells were treated for 3 days as described in the legend to Figure 4, and viable cells were determined using an 3-(4,5-dimethiazol-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (A1). PANC-1 cells were treated for 1 to 5 days as described in the legend to Figure 5, and viable cells were determined by viable cell counting (A2) PBS indicates phosphate-buffered saline; si-Ctr, control small-interfering RNA; si-Sp1, Sp1 small-interfering RNA. (B) PANC-1 cells were treated with PBS (B1), si-Ctr (B2), or si-Sp1 (B3) for 48 hours as described in the legend to Figure 4. Cells were stained with propidium iodide and were subjected to fluorescence-activated cell sorter analysis. Percentages in M1 (G1), M2 (G2/M), M3 (S), and M4 (apoptosis) are calculated. This was 1 representative experiment of 2 that produced similar results.





**Figure 10 Knockdown of transcription factor Sp1 expression effectively attenuates the *in vivo* angiogenic potential and growth of human pancreatic cancer.** FG or PANC-1 cells, FG or PANC-1 cells transfected with control small-interfering RNA (si-Ctr), and FG or PANC-1 cells transfected with Sp1 small-interfering RNA (si-Sp1) were injected into the pancreas of mice (n = 5). (A) Primary pancreatic tumors were weighed (A1, A2) PBS indicates phosphate-buffered saline. (B) The microvessel density status of PANC-1 pancreatic tumors formed by PANC-1 cells (B1), PANC-1 cells transfected with si-Ctr (B2), and PANC-1 cells transfected with si-Sp1 (B3) was determined by using CD31 staining. \*  $P < 0.05$  in a comparison between the treated and respective control groups.

To summarize the results, Sp1 expression correlated with VEGF expression and MVD in pancreatic tumor patient specimen. Sp1 protein can be knocked down specifically by using Sp1 specific siRNA oligo. Knockdown Sp1 expression in human pancreatic cancer cells leads to their losing of angiogenic potential. All the experimental results indicated that Sp1 has a key function in pancreatic cancer angiogenesis.

## DISCUSSION

In this chapter, evidence from clinic and experiment were provided to show that transcription factor Sp1 is an important regulator of human pancreatic cancer angiogenesis. Especially, the Sp1 protein expression level was correlated directly with VEGF expression level and MVD status in human pancreatic cancer. Specifically knockdown human Sp1 expression led to the inhibition of pancreatic cancer angiogenesis and tumor growth *in vivo*. In addition, the anti-angiogenic activity of knock down Sp1 directly resulted in the down-regulation of Sp1 downstream pro-angiogenic molecules VEGF, PDGF, and EGFR. The experimental results provided strong and important evidence to support the point that Sp1 is the key angiogenic regulator in human pancreatic cancer and would have significant implications in pushing the understanding of pancreatic cancer angiogenesis and important directional logical designing of effective anti-angiogenesis therapies for the disease.

As it has been discussed previously, angiogenesis has important role in both the maintaining of growth and metastasis pancreatic cancer. Both scientific researchers and clinicians have more and more interest in pancreatic cancer angiogenesis. The anti-angiogenic therapy is becoming a hot field and more scientific research results were translated to pre-clinic and clinic processes. Studies have demonstrated that anti-angiogenic therapies suppress tumor growth in pancreatic cancer mouse models [130, 144-147]. It has becoming

wildly recognized that human pancreatic cancer has elevated angiogenesis in which a distorted local balance of multiple pro-angiogenic and anti-angiogenic factors is involved [58, 84, 86, 87, 148, 149]. The imbalance is the predomination of angiogenic factors over angiogenic inhibitors. Among the growing list of angiogenic factors, all of the VEGF isoforms were reported to be important for the pancreatic tumor growth and metastasis [58, 84]. Additionally, other angiogenic factors are also over expressed in pancreatic cancer cells, such as EGF, HGF, TGF $\alpha$ , PDGF, FGFs (FGF-1, FGF-2, and FGF-5) and platelet-derived endothelial cell growth factor. The K-*ras* mutations and over-expression of HIF-1 $\alpha$ , thrombospondin-1, thymidine phosphorylase, and cathepsins B and L [58, 84] are also important contributing factors. Studies have demonstrated that expression of these factors or the combination of these factors or the combinations of them with other factors correlated with elevated vascular formation. The poor prognosis of pancreatic cancer patients under these pathological situations was also reported [58, 84]. Interference of the expression and the substance function of these factors have significant influences on angiogenesis and xenograft pancreatic tumor growth in mouse models [124, 150]. However simultaneously manipulating several target molecules remains a big challenge in clinic.

It has been demonstrated that Sp1 is constitutive activated in pancreatic cancer cells. The constitutive Sp1 activity is essential for constitutive, inducible VEGF expression [58, 61, 88, 108, 134, 151]. The current experimental results

demonstrated that over-expression of Sp1 is correlated with MVD status in human pancreatic cancer tissue. Knockdown Sp1 protein expression with the siRNA oligo significantly inhibits the tumor angiogenic potential. The Sp1 expression knocking down produced anti-angiogenic effect. That is consistent with down-regulated expression of several angiogenic signaling molecules by knockdown Sp1 expression. These molecules are in the signaling pathways that can regulate pancreatic cancer angiogenesis [60, 108, 109]. All these experimental results suggested that the transcriptional regulation is the underlying mechanism of over-expression of various angiogenic factors that collectively regulate pancreatic cancer angiogenesis [58, 88, 150].

The current study had also demonstrated that knockdown of Sp1 expression significantly reduced the angiogenic potential of human pancreatic cancer cells *in vitro* and led to the angiogenic phenotypic change *in vivo*. All of the evidence indicates that Sp1 plays an important role in pancreatic cancer angiogenesis regulation.

## **CHAPTER II**

### **Mithramycin Based Anti-angiogenic Therapy of Pancreatic Cancer in Mouse Model**

#### **INTRODUCTION**

##### **HUMAN PANCREATIC CANCER THERAPEUTICS**

In the past twenty to thirty years, pancreatic cancer patients' cases have increased greatly. It is ranked as the fourth leading cause of cancer related death in the North America currently. Pancreatic cancer has a high case: fatality ratio and the etiology are still poorly explored although various approaches have been used to identify its origin [152]. Pancreas cancer patient is almost incurable. The overall survival (OS) rate is less than 4% [153]. If the tumor is detected in resectable localization and period, it has highly curable ratio. However, pancreatic cancer patients found in the stage are less than 20%. Recently medical imaging technology, such as positron emission tomographic (PET) scans, magnetic resonance imaging (MRI) scans, high resolution endoscopic ultrasound examination, and spiral computed tomographic (CT) scans has been improved. Together with the improving prevention, the pancreatic cancer diagnosis has been moving forward.

Besides the remaining problem of its diagnosis, pancreatic cancer is highly resistant to chemotherapy and radiation therapy. Surgery resection is still the primary choice when it is feasible [154-156]. There has been conflicting results reported by different researches on postoperative therapy. The management of patients after surgery remains to be another important question to be answered [157-159].

Currently, it is urgent to identify new targets for treating pancreatic cancer patients effectively. To fulfill the task, it is important to understand pancreatic cancer development and progression. There are average sixty three genetic alternations in pancreatic cancer patient. All of these genes that regulate angiogenesis, growth, and metastasis could be the potential targets [84, 85, 155-157, 160-162]. Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) were considered to be the most important angiogenic factors. In pancreatic cancer epidermal growth factor (EGF) and its receptor (EGFR), insulin-like growth factor I (IGF-I) and its receptor, hepatocyte growth factor and its receptor Met were also considered to be important to angiogenesis [58, 84, 163]. Pancreatic cancer angiogenesis is correlated with these of angiogenic factors' expression. Over-expression of VEGF has been reported to be correlated with microvessel density (MVD) in human pancreatic cancer. In mouse model VEGF-targeting therapy inhibited angiogenesis and tumor growth of pancreatic cancer [58, 84, 163]. But how these angiogenic factors could be up-regulated is to be determined [58, 84].

Not only our lab but also other group reported that VEGF expression was regulated by Sp1 in pancreatic cancer [61, 130, 151]. With the importance of VEGF in angiogenesis of pancreatic cancer, Sp1 could be an anti-angiogenic target in the treatment. Besides this, the role of Sp1 in anti-angiogenic therapeutic treatments is also to be determined. Detailed mechanism studies of these treatments would provide better understanding to the clinical outcomes. Pancreatic cancer treatment regimen might be developed systematically and specifically.

## **MITHRAMYCIN**

Mithramycin is an aureolic acid antibiotic that is produced by a genus of bacteria *Streptomyces sp* [164, 165]. Most of the commercial available mithramycin is purified from *Streptomyces argillaceus*. In the past, it was mainly used to treat hypercalcemia, the high calcium level in blood or urine, in patients with bone metastases [166, 167]. Physicians also used it in treatment of Paget disease, testicular carcinoma and leukemia [168-173]. Recently, it was reported that mithramycin might be a potential neuroprotective drug in Huntington's disease treatment [174].

Mithramycin is believed to bind to GC-rich regions in DNA and thus regulates the transcription of the downstream genes [175]. Mechanistically two mithramycin molecules bind to DNA sequence with GC-base specificity. The binding is coordinated by a divalent cation, such as magnesium or zinc ion, and is believed



to be a reversible process [176]. Recently, it was reported that mithramycin stimulated tumor necrosis factor- $\alpha$ -related apoptosis-inducing ligand, Fas ligand and tumor necrosis factor induced apoptosis in tumor cells and prevented p53-mediated transcriptional activation [177-180]. The major mechanism of mithramycin, including anti-tumor activity, is believed to inhibit Sp1 binding activity.

## **BEVACIZUMAB**

Living cells constantly need oxygen and nutrients to maintain the metabolism. More blood supply is needed by tumor cells comparing with normal cells for maintaining their higher proliferation rate [84, 181, 182]. During the tumor growth, tumor cells produce growth factors to activate angiogenesis process to trigger the formation of new blood vessels from the existing vessel. There are lots of growth factors that were demonstrated to activate this progress. Vascular endothelial growth factor (VEGF) was believed to be one of the most potent molecules for the growth of pancreatic tumor [116, 117]. Besides this, it was also demonstrated that VEGF promotes microvessel formation around tumor [116]. Moreover, it can also increase the blood vessel permeability to provide more nutrients for tumor cells growth and metastasis [117].

Bevacizumab (Avastin™) was developed and marketed by Genentech. It is believed that Bevacizumab neutralizes soluble VEGF to inhibit new blood vessel formation and causes the destruction of the existing vessels [89, 183]. Thus the

blood supply to tumor cells would be blocked and tumor cells growth would slow down. Bevacizumab was approved by U.S. Food and Drug Administration (FDA) in 2004. It is the first approved biological therapy drug for combination chemotherapy in several types of tumors. Under the instruction of National Institutes of Health, Genentech cooperated with the National Cancer Institute (NCI) to develop of clinical practice of Bevacizumab in cancer treatment. Bevacizumab is humanized murine monoclonal antibody that binds to and inhibits VEGF [89]. A monoclonal antibody is a targeting protein produced by hybridoma.

Use Sp1 as a potential anti-angiogenic target for effective targeting therapy in pancreatic cancer and its role in anti-angiogenic drug currently in clinic trial have not been explored till then. In this chapter, I sought to determine whether mithramycin has any effect on pancreatic cancer angiogenesis and if so, what the corresponding molecular mechanism is.

## **MATERIAL AND METHODS**

### **Chemicals and Reagents Information**

Mithramycin (1 mg per vial crystal powder) was purchased from Sigma Chemical Co (St. Louis, MO). It was dissolved in sterile water and diluted for actual use. Bevacizumab (25 mg/ml) was purchased from Genentech, Inc. For animal experiments, mithramycin (0.1–0.4 mg/kg body weight) and Bevacizumab (25–100 µg per mouse) were given by i.p. injection twice a week or as indicated otherwise.

### **Animals Information and Animal Care**

Female athymic BALB/c nude mice were purchased from The Jackson Laboratory. The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used when they were 8 weeks old. The animals were maintained in M D 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, U.S. Department of Health and Human Services, and NIH.

### **Cell Lines and Culture Conditions Information**

The human pancreatic adenocarcinoma cell lines PANC-1 and BxPC3 were purchased from the American Type Culture Collection (Manassas, VA). FG

human pancreatic adenocarcinoma cells were established by Veziridis et al [137]. The cell lines were maintained in plastic flasks as adherent monolayers in MEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and a vitamin solution (Flow Laboratories).

### **Immunohistochemical Analysis and Tumor MVD Quantification Protocols**

For CD31 staining, frozen tissue sections (5  $\mu$ m thick) were fixed in acetone. Endogenous peroxidase in the specimens was blocked using 3% hydrogen peroxide in PBS for 12 min. The specimens were incubated for 20 min at room temperature in a protein-blocking solution consisting of PBS (pH, 7.5) containing 5% normal horse serum and 1% normal goat serum and then incubated overnight at 4°C in a 1:100 dilution of monoclonal goat anti-CD31 (PECAM1-M20), polyclonal rabbit anti-Sp1, or polyclonal rabbit anti-VEGF antibodies (Santa Cruz Biotechnology). The specimens were then rinsed and incubated with peroxidase-conjugated anti-goat or anti-rabbit IgG for 1 hour at room temperature. Next, slides were rinsed with PBS and incubated with diaminobenzidine (Research Genetics) for 5 min. Frozen sections of the specimens were then washed twice with distilled water, counterstained with Mayer's hematoxylin (Biogenex Laboratories), and washed once each with distilled water and PBS. Sections were mounted on the slides using Universal Mount (Research Genetics), and the slides were examined under a bright-field microscope. A CD31-positive, Sp1-positive reaction was indicated by a reddish-brown precipitate in the cytoplasm or

nuclei, respectively. For quantification of tumor MVD, vessels on each section were counted in five high-power fields (magnification, x200 [x20 objective and x10 ocular]) as described previously [184].

### **Western Blot Analysis Protocol**

Whole-cell lysates were prepared from human pancreatic cancer cell lines and tissues [174]. Standard Western blotting was done using polyclonal rabbit antibodies against human and mouse Sp1, VEGF, PDGF and EGFR (Santa Cruz Biotechnology) and the anti-rabbit immunoglobulin G (IgG) antibody, a horseradish peroxidase-linked F(ab')<sub>2</sub> fragment obtained from a donkey (Amersham). Equal protein-sample loading was monitored by probing the same membrane filter with antibodies against anti- $\beta$ -actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [60]. The probe proteins were detected using the Amersham enhanced chemo-luminescence system according to the manufacturer's instructions.

### **Sp1 Promoter Constructs Information and Analysis of Sp1 Promoter Activity with Luciferase Assay**

The minimal Sp1 promoter reporters in pGL3 luciferase constructs were generated and used as described previously [59, 61]. In order to examine the transcriptional regulation of the Sp1 promoters by Bevacizumab and mithramycin, PANC-1 cells were seeded to about 80% confluence in six-well plates (in

triplicate) and transiently transfected with 0.6 µg of minimum Sp1 reporter plasmids and 0.3 µg of effectors expression plasmids as indicated in each experiment using Lipofectamine (Invitrogen) according to the manufacturer's instructions. The reporter luciferase activity was measured 48 hours later using a luciferase assay kit (Promega). Promoter activity was normalized according to the protein concentration as described previously [59].

### **Chromatin Immunoprecipitation Protocol**

Chromatin was prepared from cells and tumors as described previously [59]. Chromatin immunoprecipitation (ChIP) assay was done using the Chromatin Immunoprecipitation Assay Kit (Upstate Cell Signaling Solutions) according to the manufacturer's instructions. Briefly, DNA cross-binding proteins were cross-linked with DNA and lysed in SDS lysis buffer. The lysate was sonicated to shear DNA to 200 to 500 bp. After pre-clearing with a salmon sperm DNA/protein A agarose–50% slurry for 30 min at 4°C, chromatin samples were immunoprecipitated overnight with no antibody or an anti-Sp1 antibody (PEP2). The region between –224 and –53 bp of the *Sp1* promoter was amplified using the following primers: sense, 5'-caggcacgcaacttagtc-3', and antisense, 5'-gtaaggaggaggagcag-3'. PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

### **Statistical analysis**

Each experiment was done independently at least twice with similar results; one representative experiment is presented. The significance of the *in vitro* data was determined using Student's *t* test (two-tailed), whereas the significance of the *in vivo* data was determined using the two-tailed Mann-Whitney *U* test. For the *in vivo* experiments, the overall survival duration was calculated using the Kaplan-Meier method. The log-rank test was used to compare the survival duration between groups.  $P < 0.05$  was deemed significant.

## **RESULTS**

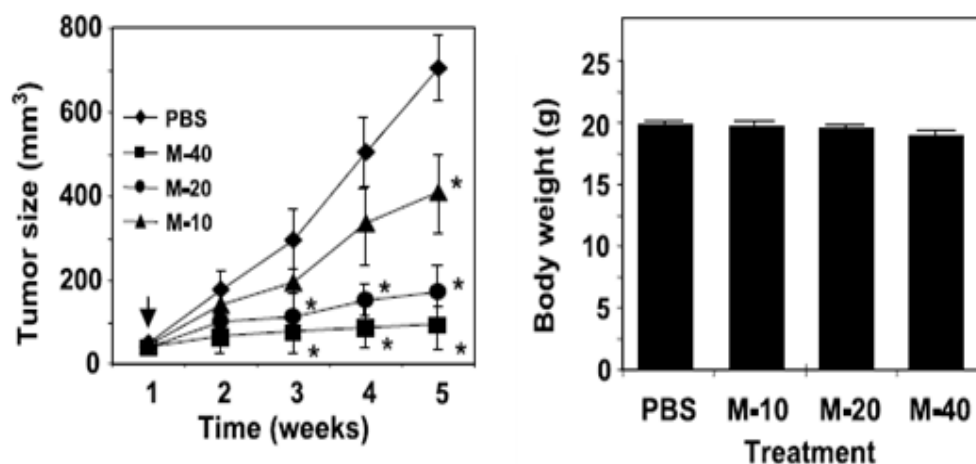
### **PART 1. MITHRAMYCIN TREATMENT ON HUMAN PANCREATIC CANCER ANGIOGENESIS**

#### **Mithramycin treatment inhibits human pancreatic tumor growth in xenograft mouse models**

As it was introduced, mithramycin, as a Sp1 inhibitor, can inhibit Sp1 protein expression and interpose Sp1 recruitment to Sp1 sites. But whether it can inhibit pancreatic tumor growth *in vivo* is still unknown.

First, a set of dose-response experiments was performed. BxPC-3 cells were injected subcutaneously into nude mice. When the tumors reached 4 mm in diameter, animals were given mithramycin in different doses (0.1, 0.2, and 0.4 mg/kg) via intraperitoneal injection twice weekly. PBS was injected into a group of animals that used as controls. It was observed that treatment with mithramycin produced dose-dependent antitumor activity (Fig. 11).



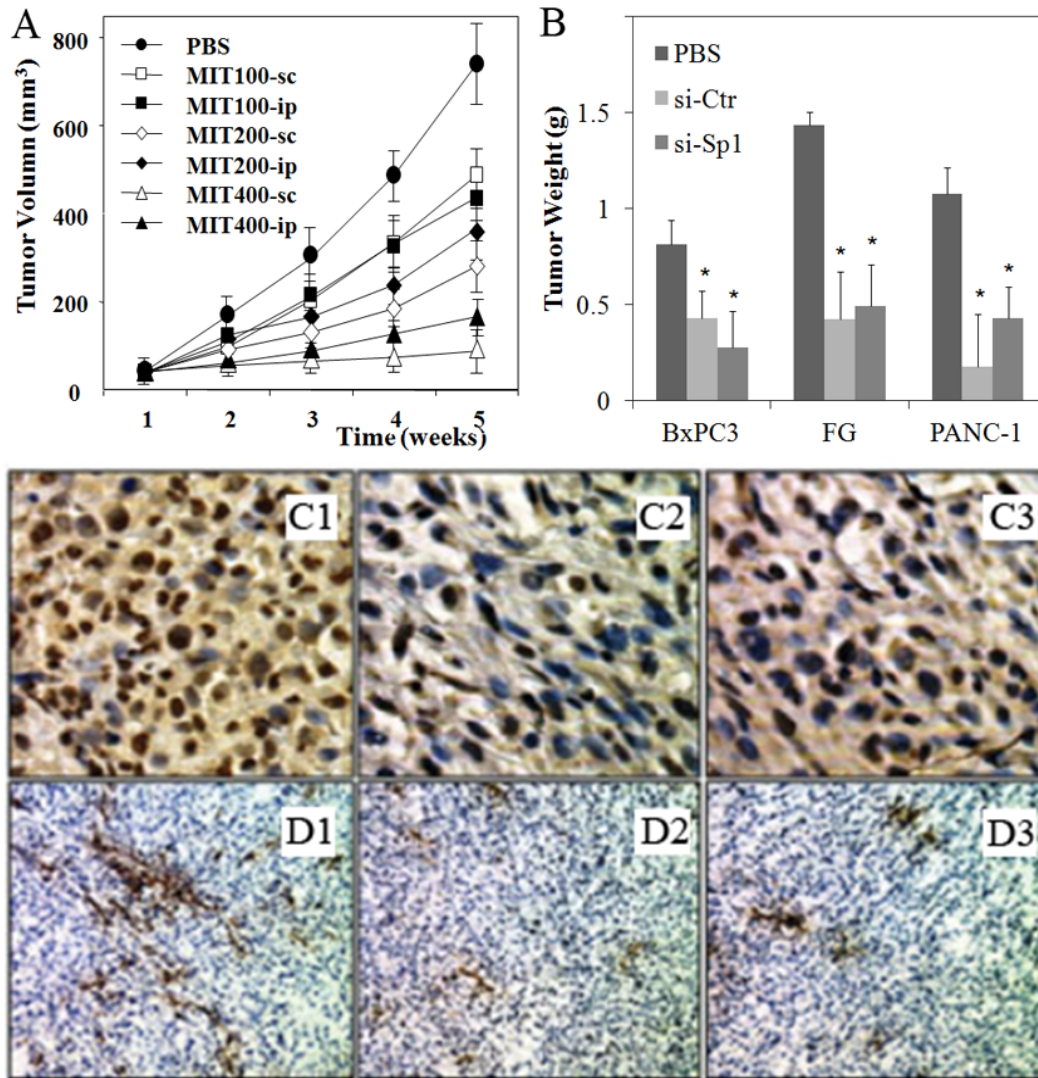


**Figure 11 Dose-dependent antitumor effects of mithramycin in xenograft models of human pancreatic cancer.** BxPC3 cells were injected into the subcutis of nude mice ( $n = 5$ ). When tumors reached around 4 mm in diameter, the animals received different doses of mithramycin [0.10 (*M-10*), 0.20 (*M-20*), and 0.40 (*M-40*) mg/kg] via i.p. injection twice a week. Tumors were measured once every week, and at each measurement, the mean  $\pm$  SD tumor volume in the five mice in each group was calculated. The control mice and mice that received mithramycin were weighed at the time of experiment termination. *Columns*, mean weights; *bars*, SD. \*  $P < 0.01$  in a comparison between the treated and respective control groups

Then I sought to determine whether different administer and slow release of mithramycin would have the same effect on xenograft tumors. BxPC3 cells were inoculated to mice subcutis. When the tumors reached 4 mm in diameter, the animals were gave different doses of mithramycin (0.12 mg/kg, 0.25 mg/kg, and 0.50 mg/kg) by subcutaneous or intraperitoneal injection twice weekly. It was observed that mithramycin treatment by intraperitoneal and subcutaneous administration can both produce dose-dependent antitumor activity (Fig. 12A).

Next, similar experiments were performed using orthotopic mouse models. Pancreatic xenograft tumors were formed in mice pancreas as described in Material and Methods. Same dose and frequency of mithramycin were administered. It was found that treatment with mithramycin (0.25 mg/kg by intraperitoneal and subcutaneous administration) significantly inhibited the growth of BxPC-3, FG, and PANC-1 formed tumors (Fig. 12B).

Furthermore, a histopathology examination was performed using pancreatic tumor specimens that were obtained from control mice and from mice that received low doses of mithramycin, because no tumors formed in mice that received high doses of mithramycin. Significant inhibition of Sp1 expression in tumor specimens from mice that received mithramycin compared with the control mice was observed (Fig. 12C), which was consistent with reduced MVD (Fig. 12D).



**Figure 12 Suppression of human pancreatic cancer growth by mithramycin in xenograft models** (A) BxPC-3 cells were injected into the subcutis of groups of mice (n = 5 each). When tumors reached approximately 4 mm in greatest dimension, animals received injections of PBS or mithramycin (0.12 mg/kg, 0.25 mg/kg, and 0.50 mg/kg) subcutaneously (s.c.) or intraperitoneally (i.p.) twice weekly. The tumors were measured once weekly; and, at each measurement, the mean  $\pm$  standard deviation tumor volume was calculated in each group of mice. (B) BxPC-3, FG, and PANC-1 cells were injected

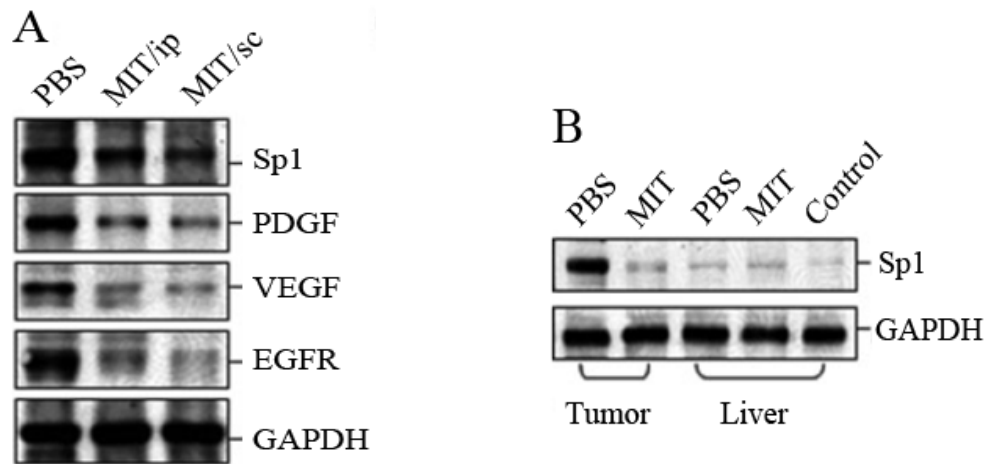
into the pancreas of mice ( $n = 5$ ). Two weeks after tumor injection (when tumors measured approximately 4 mm in greatest dimension), mice received i.p. or s.c. injections of mithramycin (0.25 mg/kg) twice weekly. PBS injection was used as a control. The resulting primary pancreatic tumors were weighed 45 days after the injection. (C) Sp1 expression was determined in PANC-1 tumor tissues collected from mice that received treatments with PBS (C1), mithramycin i.p. (C2), or mithramycin s.c. (C3) using immunohistochemistry. (D) Microvessel density status was assessed in PANC-1 tumor tissues collected from mice that received treatment with PBS (D1), mithramycin i.p. (D2), or mithramycin s.c. (D3) by using CD34 staining. This was 1 representative experiment of 2 that produced similar results. \*  $P < 0.05$  in a comparison between the treated and respective control groups.

### **Gene expression analysis of mithramycin treated tumors**

Because mithramycin treatment produced dose-dependent tumor growth inhibition effect, I then sought to determine which potential angiogenic molecules were also affected in addition to Sp1, which were related to the anti-angiogenic effect.

Western blot analysis was performed using total protein lysates extracted from the PANC-1 tumor tissue specimens that collected from mice that received treatment with PBS or mithramycin. Mithramycin suppressed the expression of Sp1 and its downstream molecules VEGF, EGFR, and PDGF (Fig. 13A), which was consistent with reduced MVD. It is noteworthy that the expression of Sp1 in normal tissues, including the liver, was not suppressed significantly (Fig. 13B).

The results of this part suggested that mithramycin preferentially inhibits Sp1 expression in growing tumors and has potent anti-pancreatic cancer activity. It was demonstrated that treatment with mithramycin inhibits Sp1 expression and down-regulates the downstream targets that are key to the angiogenesis of human pancreatic cancer as the consequence.



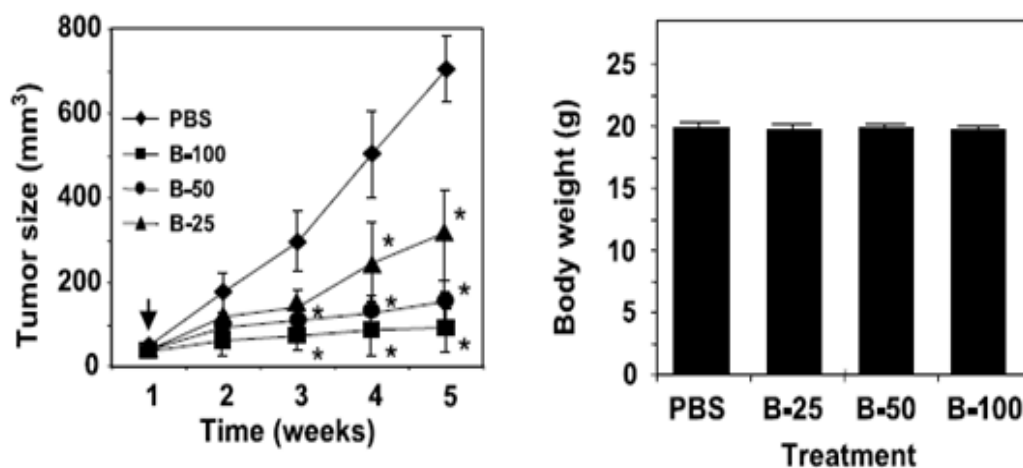
**Figure 13 Preferential inhibition of transcription factor Sp1 expression in growing pancreatic tumors and anti-angiogenic effects.** When BXPC-3 tumors reached approximately 4 mm in greatest dimension, the animals received 2 subcutaneous (s.c.) injections of phosphate-buffered saline (PBS) (controls) or mithramycin A (mithramycin) (0.25 mg/kg). Total protein lysates were extracted from the tumors, from the livers of mice that received treatment with PBS or mithramycin 24 hours after injections, and from the livers of untreated mice (control). (A) Western blot analysis was used to determine the expression of Sp1, vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), and platelet-derived growth factor (PDGF) in tumors that were growing in mice that had received 2 injections of PBS or mithramycin either intraperitoneally (mithramycin/i.p.) or mithramycin/s.c.) GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase. (B) The levels of Sp1 expression were determined in both tumors and normal tissues by using Western blot analysis.

## **PART 2. ROLE OF SP1 IN CURRENT ANTI-ANGIOGENIC THERAPY IN PANCREATIC CANCER**

### **Bevacizumab and mithramycin combination produced synergistic antitumor effects in human pancreatic cancer mouse models**

Several researches have reported that Sp1 is the key for VEGF expression. VEGF plays an important role in regulating pancreatic cancer angiogenesis. However, whether Sp1 signaling was altered and what role that Sp1 has in anti-VEGF anti-angiogenic therapy is unknown.

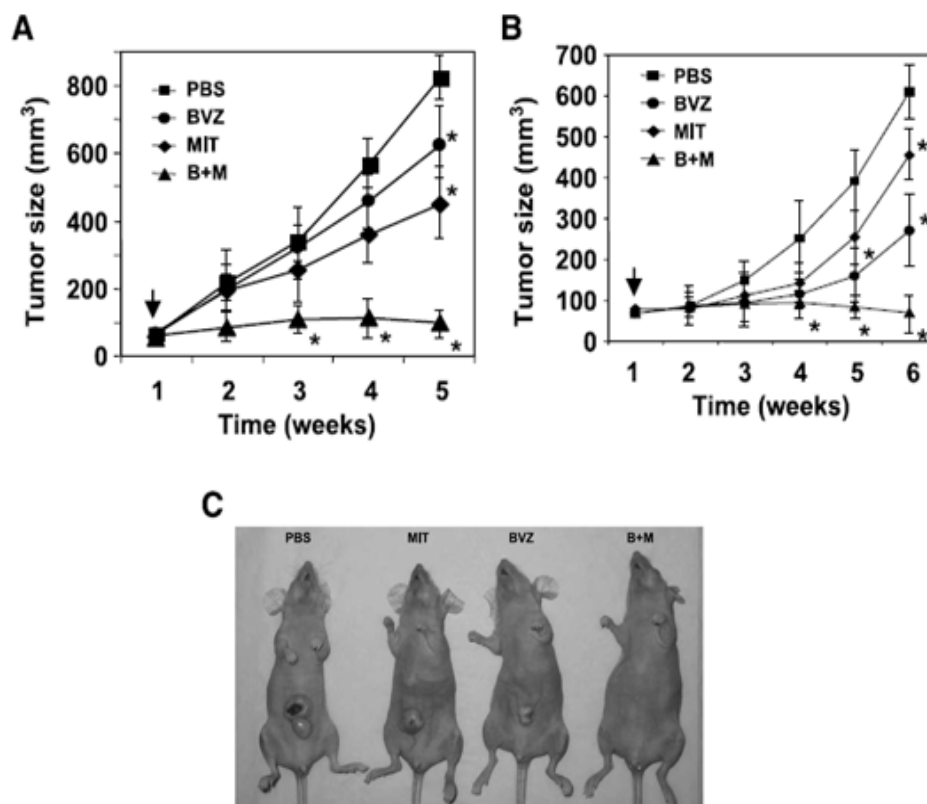
To address these questions, the anti-tumor effect of anti-VEGF monoclonal antibody Bevacizumab was tested. Specifically, a dose-response experiment was first performed. BxPC3 cells were injected into subcutis of nude mice. When the resulting tumors reached 4 mm in greatest dimension, Bevacizumab (25, 50, and 100 µg; Fig. 14) was given to the animals via i.p. injection twice a week. PBS was injected to a group of animals as controls. It was found that treatment with Bevacizumab produced dose-dependent antitumor activity. The mice that received Bevacizumab did not have significant body weight loss (Fig. 14). The experimental result suggested that Bevacizumab did not have any systemic side effects at the doses given.



**Figure 14 Dose-dependent antitumor effects of Bevacizumab in xenograft models of human pancreatic cancer.** BxPC3 cells were injected into the subcutis of nude mice ( $n = 5$ ). When tumors reached around 4 mm in diameter, the animals received different doses of Bevacizumab [25 (B-25), 50 (B-50), and 100 (B-100)  $\mu$ g] via i.p. injection twice a week. Tumors were measured once every week, and at each measurement, the mean  $\pm$  SD tumor volume in the five mice in each group was calculated. The control mice and mice that received were weighed at the time of experiment termination. *Columns*, mean weights; *bars*, SD. \*  $P < 0.01$  in a comparison between the treated and respective control groups.



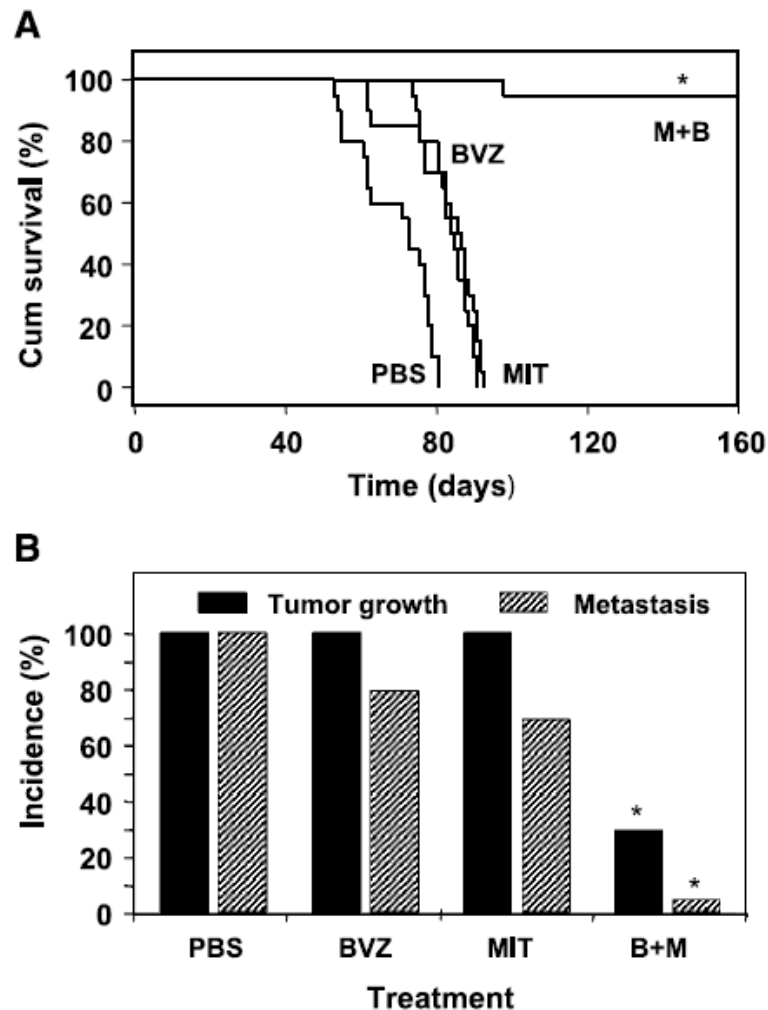
Next, I sought to determine the treatment effect of the combination of the Bevacizumab and mithramycin at the dose that has minor anti-tumor effect. Experiments were performed using both BxPC3 and PANC-1 tumor cell models. Specifically, PBS, Bevacizumab (25  $\mu$ g), mithramycin (0.10 mg/kg), or B + M were given to a group of nude mice. Consistent with last experimental result, administration of Bevacizumab or mithramycin alone produced minor antitumor activity. In contrast, administration of B + M produced synergistic antitumor activity without any observed systemic side effects (Fig. 15). Also, the mice body weights that received B + M is similar to the control mice that received PBS. Therefore, the use of B + M produced higher antitumor activity than the use of Bevacizumab or mithramycin alone did and no increased toxicity observed. These experimental results suggested that B + M treatment has a significant therapeutic benefit in pancreatic cancer mouse model.



**Figure 15 Synergistic antitumor effects of treatment with Bevacizumab and mithramycin in xenograft models of human pancreatic cancer.** Both (A) BxPC3 and (B) PANC-1 cells were injected into the subcutis of groups of mice ( $n = 5$ ). Specifically, animals received injections of PBS (controls), Bevacizumab (25  $\mu$ g), mithramycin A (0.10 mg/kg), or B + M. Tumors were measured once every week, and at each measurement, the mean  $\pm$  SD tumor volume in the five mice in each group was calculated. A and B, \*  $P < 0.01$  in a comparison between the treated and respective control groups. C, representative tumor sizes in each group of BxPC3 model mice.

### **Bevacizumab and mithramycin produced prolonged survival in orthotopic xenograft model of human pancreatic cancer**

PANC-1 cells were injected into the pancreas of nude mice and then treatments were given to them as described in Figure 15. Animal survival was monitored daily. The experiment was terminated at 160 days after tumor-cell injection. It was found that Bevacizumab or mithramycin alone treatment produced a slightly increased survival duration comparing with PBS group. However, the B + M combination treatment produced longer survival duration when comparing with the other three groups of mice and the difference was statistically significant (Fig. 16A). In addition to this, the decreased incidence of tumor growth in the pancreas and of metastasis in the liver and/or other organs was found in the B + M combination treatment group (Fig. 16B). The decrease is statistically significant.



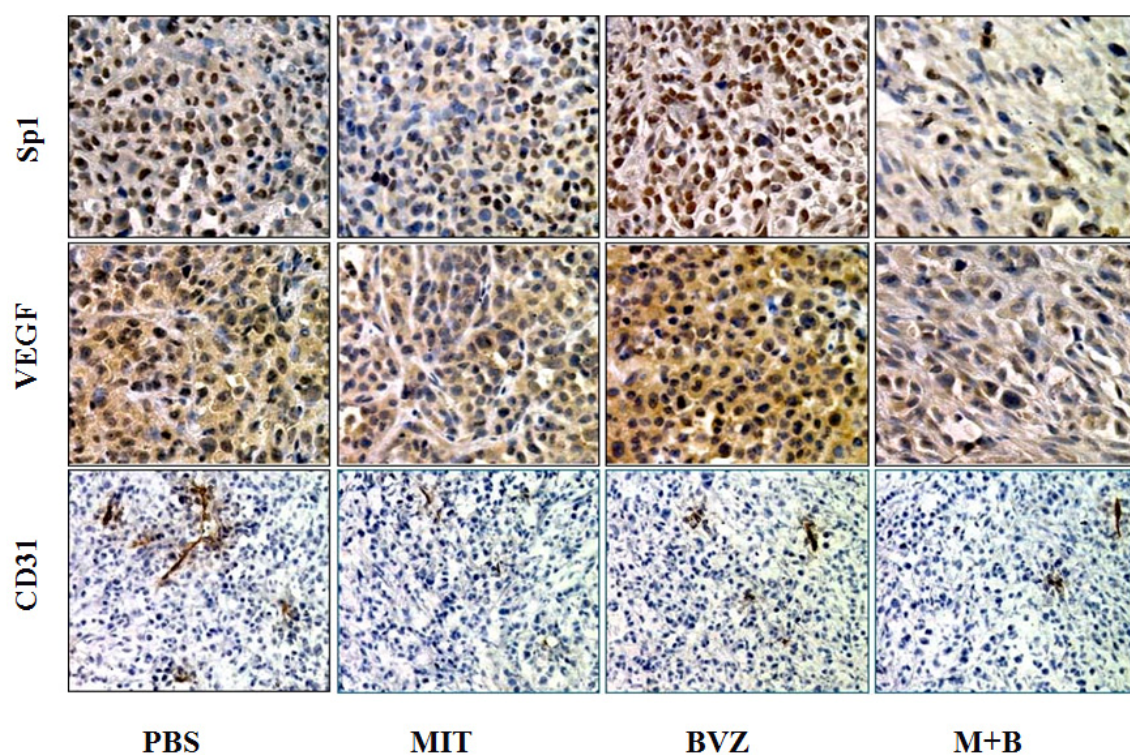
**Figure 16 Prolonged survivals of mice that received treatment with Bevacizumab and mithramycin in xenograft models of human pancreatic cancer.** PANC-1 cells were injected into the pancreas of nude mice (17–20 mice per group). The mice received treatment as described in Fig. 15. The entire experiment was terminated 120 days after tumor-cell injection. *A*, animal survival was monitored daily until the termination of the experiment. *Cum*, cumulative. *B*, tumor growth in the pancreas and metastasis in the liver and/or other organs were evaluated and expressed as the incidence (%). \*  $P < 0.01$  in a comparison between the treated and respective control groups.

### **Bevacizumab treatment up-regulates Sp1 and VEGF expression and its reversal by treatment with mithramycin**

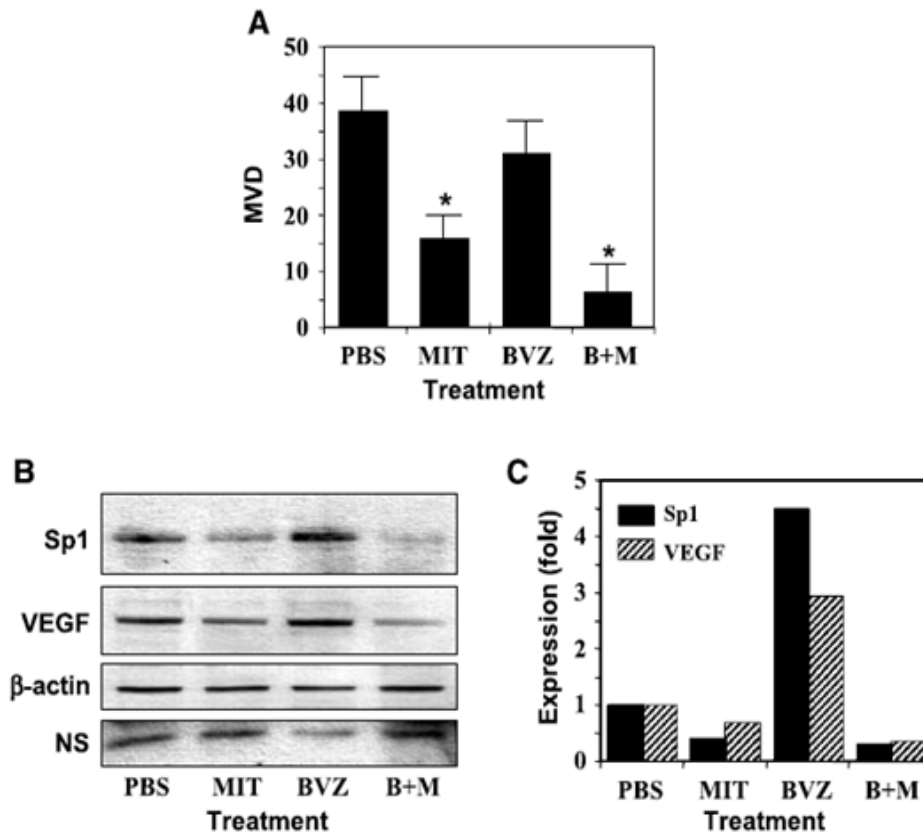
In this experiment, genes expression analysis was used to determine the mechanism of the synergistic effect of B + M combination treatment.

Specifically, PANC-1 tumor tissues were harvested from mice that received treatment with PBS, Bevacizumab, mithramycin, or B + M. The tissue specimen were then stained using specific antibodies against Sp1, VEGF and CD31 respectively. Representative pictures from each group were shown in Figure 17. As it shows, Bevacizumab treatment increases Sp1 and its downstream molecule VEGF expression. However, treatment with mithramycin suppressed Sp1 and VEGF expression, which was consistent with reduced MVD (Fig. 17 and 18 A), whereas treatment with Bevacizumab at the low dose alone did not significantly reduce MVD, which was consistent with increased Sp1 expression.

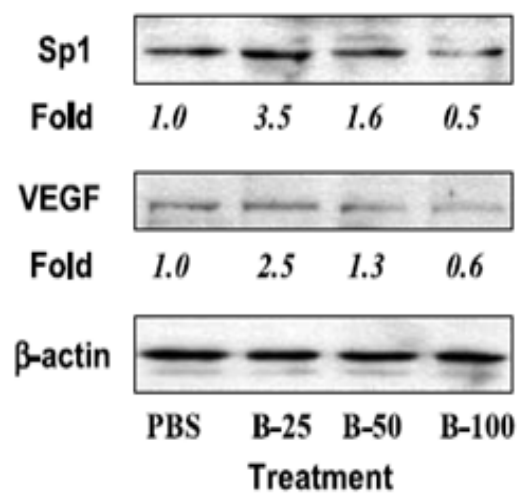
Next western blot analysis was performed. Tumor tissue specimens were collected from mice that received treatment with PBS, Bevacizumab, mithramycin, or B + M. Total protein lysates were extracted and run western blot analysis As shown in Fig. 18B and C. Consistent with the immunohistochemistry staining results, the expression of Sp1 and its downstream angiogenic factor VEGF were up-regulated by Bevacizumab treatment.



**Figure 17 Gene expression analyses of tumor from mice that received treatment with Bevacizumab and mithramycin in xenograft models of human pancreatic cancer.** Sp1, VEGF expression were determined in PANC-1 tumor tissues collected from mice that received treatments in the experiment described in Figure 15 using immunohistochemistry. MVD status was assessed by using CD31 staining.



**Figure 18 Analysis of MVD and gene expression of tumor from mice that received treatment with Bevacizumab and mithramycin in xenograft models of human pancreatic cancer.** The tumor tissues described in Fig. 15 were collected and processed as described in Materials and Methods. A, MVD was quantitated according to CD31 staining. B, total protein lysates were harvested from tumor tissues and the level of protein expression in them was determined using Western blot analysis. Equal protein-sample loading was monitored by probing the same membrane filter with an anti- $\beta$ -actin antibody. NS, nonspecific band. C, the levels of Sp1 and VEGF expression were quantitated and expressed as fold change. \*  $P < 0.01$  in a comparison between the treated and respective control groups.



**Figure 19 Analysis of Sp1 and VEGF expression in tumor tissues.** The tumor tissues described in Fig. 14 were collected and western blot analysis was done using specific antibodies against Sp1, VEGF, and  $\beta$ -actin.



These data indicated that the Bevacizumab neutralizes circulating VEGF and Sp1 expression may be up-regulated through the positive feedback loop, which may lead to increased VEGF expression in cell. However, because of the initially decreased VEGF neutralized by Bevacizumab, the MVD levels may be decreased for some degree. However, Sp1 up-regulation may also activate the other downstream angiogenic protein besides VEGF. The over-expression of these angiogenic proteins might trigger the tumor's resistance to Bevacizumab treatment.

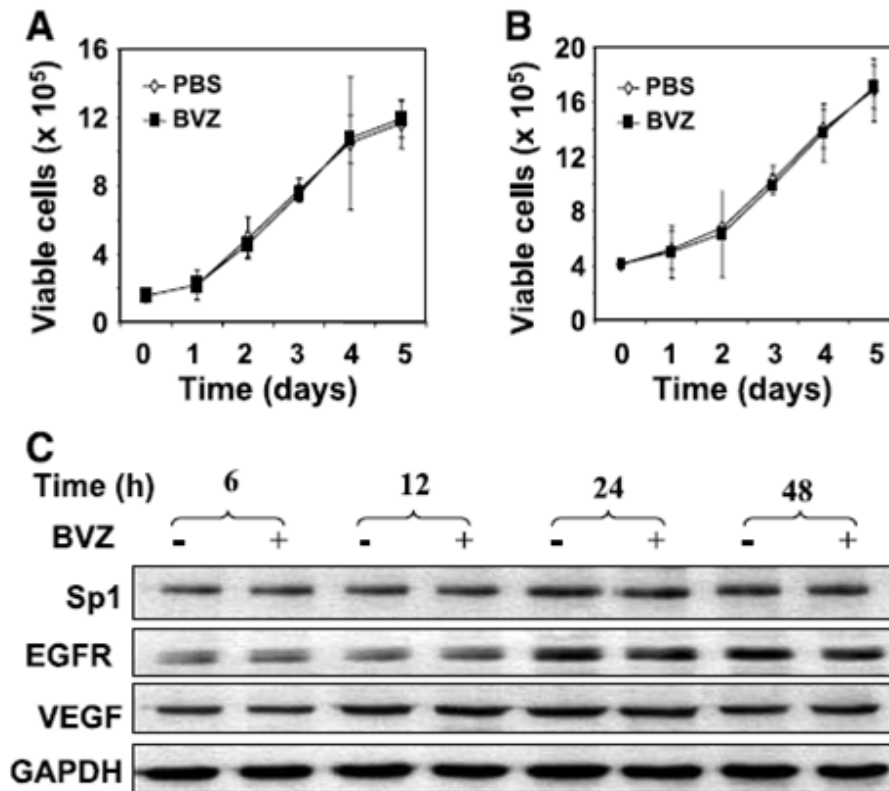
As it was shown, low dose of Bevacizumab (25  $\mu$ g) treatment that activates Sp1 expression does not have significant antitumor activity. Expression level of Sp1 in tumors harvested from mice that received high dose of Bevacizumab (100  $\mu$ g) treatment, which had a significant antitumor activity, was determined. The western blot result was shown in Fig. 19. Sp1 protein level was found to be decreased by the 100  $\mu$ g of Bevacizumab treatment. However, Sp1 expression up-regulation by the 25  $\mu$ g of Bevacizumab treatment was also observed. It was also found that the alteration of VEGF expression level was consistent with that of the Sp1 expression level. These data indicated that the ineffective low dose of Bevacizumab treatment could up-regulate Sp1 expression and might contributed to Bevacizumab resistance.

## **Bevacizumab and mithramycin treatment effect on the growth and gene expression in human pancreatic cancer cells**

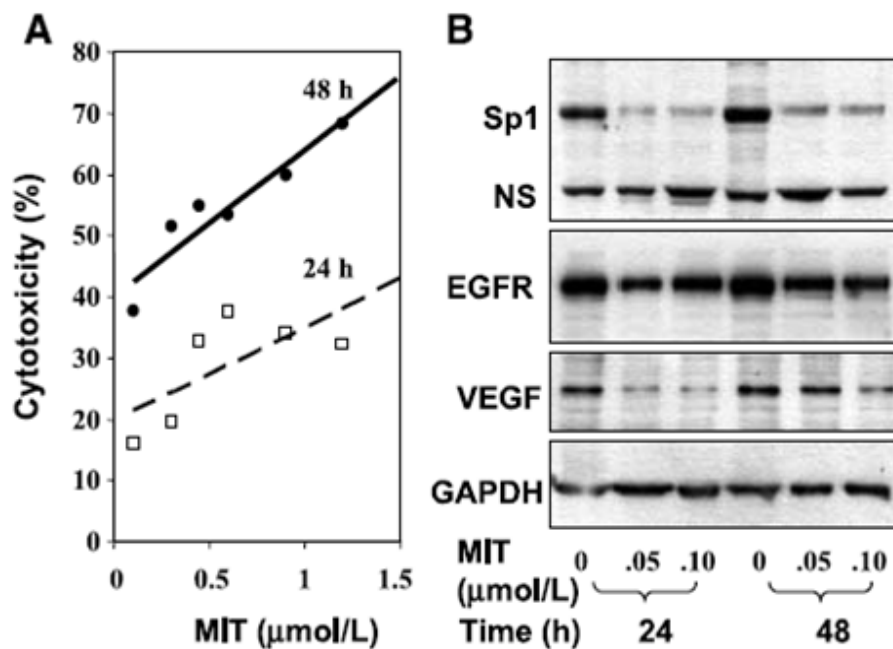
Since Bevacizumab treatment could up-regulate Sp1 and its downstream angiogenic molecules *in vivo*, I then want to determine how that affect gene expression in pancreatic cancer cells *in vitro*. PANC-1 and BxPC3 cells were incubated in 100µg/ml of Bevacizumab in culture medium. Cells were harvested in 6, 12, 24 and 48 hours and cell number were counted in day 1 to 5. As it was shown in Figure 20, Bevacizumab treatment did not affect BxPC3 cells growth (Fig. 20A) or PANC-1 cells growth (Fig. 20B) *in vitro*. The western blot was shown in Fig. 20C. Neutralization of VEGF by Bevacizumab treatment did not affect the expression of Sp1 and its downstream molecules VEGF and EGFR. As it was reported previously that Bevacizumab primarily neutralizes the circulating VEGF to block its autocrine effect on tumor angiogenesis [124, 147-149, 185]. This was confirmed by the current experimental result.

However, mithramycin treatment inhibits PANC-1 cells (Fig. 21A) growth *in vitro* in dose-dependent manner. The IC<sub>50</sub>s of 24 and 48 hours of mithramycin treatment were >1.5 µmol/L and 0.15 µmol/L respectively. But 0.05 and 0.10 µmol/L of mithramycin treatment inhibited the expression of Sp1 and its downstream molecules EGFR and VEGF in PANC-1 cells (Fig. 21B). These data indicated that mithramycin treatment inhibited tumor cells growth and down-

regulated Sp1 and its downstream molecules' expression. Whereas Bevacizumab did not have the effect.



**Figure 20** Effects of treatment with Bevacizumab on the growth of and gene expression in human pancreatic cancer cells *in vitro*. BxPC3 (A) and PANC-1 cells (B) were incubated for 1 to 5 d in medium alone or a medium containing 100  $\mu\text{g/ml}$  Bevacizumab. The viable cells were counted every 24 h. C; PANC-1 cells were incubated for 6 to 48 h in medium alone or a medium containing 100  $\mu\text{g/ml}$  Bevacizumab. Total protein lysates were harvested from the cell cultures, and the level of Sp1, EGFR, and VEGF protein expression was determined using Western blot analysis. Equal protein-sample loading was monitored by probing the same membrane filter with an anti-GAPDH antibody.

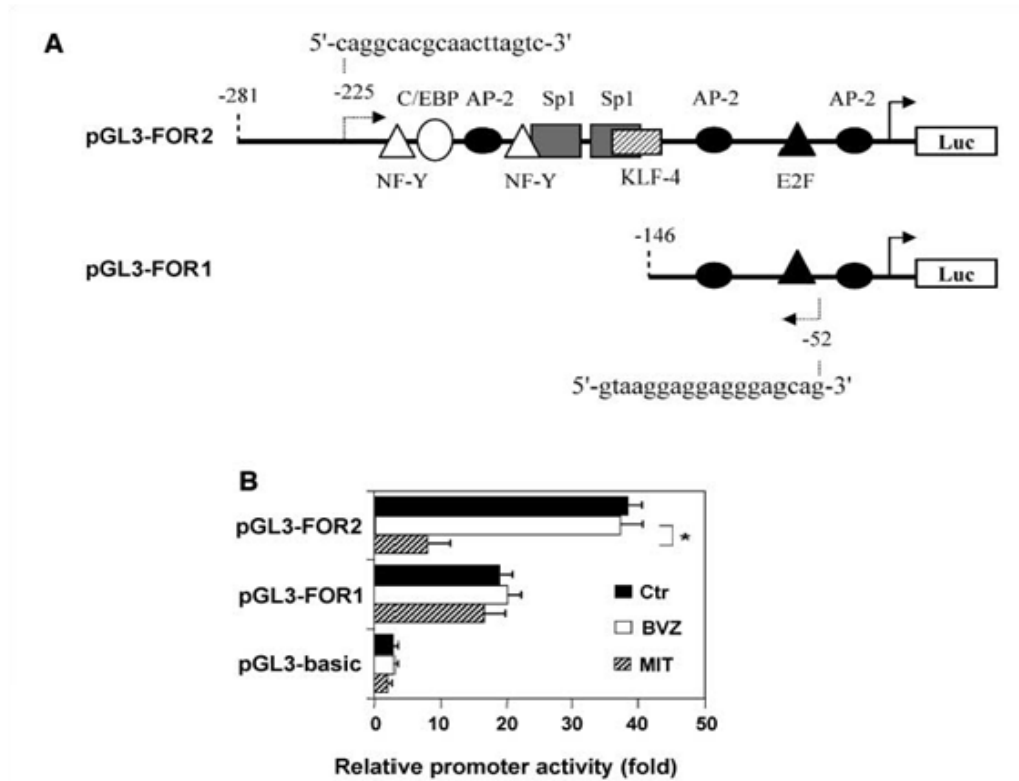


**Figure 21** Effects of treatment with mithramycin on the growth of and gene expression in human pancreatic cancer cells *in vitro*. A and B, PANC-1 cells were treated with mithramycin at concentrations ranging from 0.1 to 1.2  $\mu\text{mol/L}$  for 24 and 48 h. A, cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyltetrazolium bromide assay. B, the level of gene expression was determined using Western blot analysis. *NS*, nonspecific.

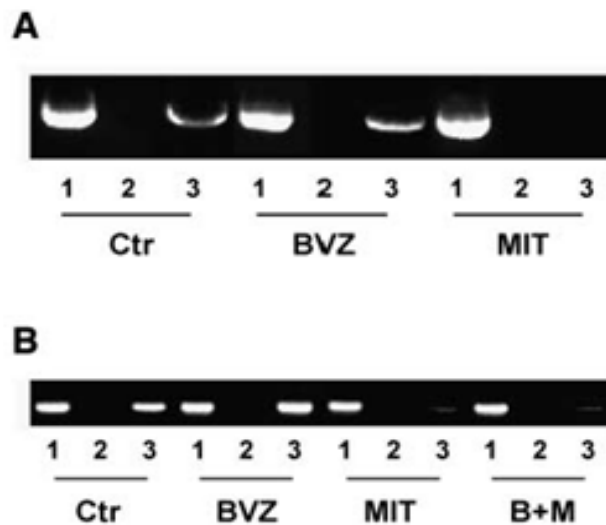
### **Effects of Bevacizumab and mithramycin treatment on Sp1 recruitment into the Sp1 promoter *in vitro* and *in vivo* in human pancreatic cancer cells**

As it was shown previously, mithramycin treatment down-regulated Sp1 protein expression and B + M combination treatment produced significant anti-tumor effect. I then sought to determine the molecular mechanism. Luciferase assay was performed to determine Sp1 promoter activity. Briefly, Sp1 promoter reporter constructs were transfected into PANC-1 cells. The cells were then incubated in 100µg/ml Bevacizumab or 0.1µmol/L mithramycin in culture medium. Mithramycin treatment suppressed Sp1 promoter activity significantly *in vitro*, but Bevacizumab treatment did not. However, Sp1 promoter activity suppression produced by mithramycin treatment was eliminated by further deletion of Sp1-binding sites in its promoter (Fig. 22 A and B).

Next ChIP assay was used to further determine the mechanism. Consistently, mithramycin treatment significantly reduced Sp1 protein recruiting onto its own promoter as shown in Figure 23 A, whereas Bevacizumab treatment (100µg/ml) did not produce this effect. Then tumors tissues harvested in the experiment described in Figure 15 were used to perform ChIP assay. Both mithramycin treatment and B + M treatment suppressed Sp1 protein recruitment to its own promoter (Fig. 23B). These data suggested that mithramycin treatment interposes Sp1 recruitment on its promoter and blocks Sp1 transcription to down-regulate Sp1 and its downstream target molecules, such as VEGF. This might disrupt the positive feedback produced by Bevacizumab.



**Figure 22 Effects of Bevacizumab and mithramycin treatment on Sp1 promoter activity *in vitro*.** A, schematic structures of the minimal Sp1 promoters. The nucleotide positions and sequences of Sp1-binding sites and PCR forward and reverse primers flanking those sites for ChIP assay are shown. B, Sp1 promoter reporter constructs were transfected into PANC-1 cells in triplicate and incubated for 12 h. The cells were then incubated for another 24 h in medium alone or a medium containing 100  $\mu$ g/ml Bevacizumab or 0.1  $\mu$ mol/L mithramycin. Total protein lysates were harvested from the cell cultures for measurement of Sp1 promoter activity using a luciferase assay kit. The relative Sp1 promoter activities were assessed, and the activity in treated groups was expressed as the fold change of that in their respective control groups.



**Figure 23 Effects of treatment with Bevacizumab and mithramycin on Sp1 recruitment into the Sp1 promoter in human pancreatic cancer cells *in vitro* and *in vivo*.** A, chromatin was extracted from PANC-1 cells that were incubated *in vitro* for 2 d in medium alone or a medium containing 100  $\mu\text{g/ml}$  Bevacizumab or 0.1  $\mu\text{mol/L}$  mithramycin. B, chromatin was extracted from tumors formed by PANC-1 cells in nude mice that received treatment as described in Fig. 15. The ChIP assay was done using a specific anti-Sp1 antibody and oligonucleotides flanking the Sp1 promoter regions containing Sp1-binding sites. *Lane 1*, input chromatin DNA; *lane 2*, chromatin DNA with control IgG; *lane 3*, chromatin DNA with anti-Sp1 antibody. *Ctr*, control.



In this part, it was found that treatment with ineffective low dose of Bevacizumab up-regulated Sp1 expression. This positive feedback is then positive regulate the expression of various angiogenic factors, including VEGF. Mithramycin treatment inhibited Sp1 expression and repressed VEGF expression and tumor angiogenesis. Low doses of B + M combination treatment produced a synergistic anti-angiogenic effect (super additive at least). This effect was correlated with Sp1 suppression activity and Sp1 downstream target molecules down-regulation. These experimental findings suggested that Bevacizumab block VEGF signaling *in vivo* may triggers the positive feedback to up-regulate p1 expression. This feedback might leads to the up-regulation of multiple angiogenic factors including VEGF in turns. In this way, Bevacizumab treatment neutralizes VEGF and may lead to compensatory up-regulation pathways, such as transcription factor Sp1, which may lead to drug resistance as consequence. However, mithramycin treatment interfere Sp1 recruitment onto its promoter and inhibit Sp1 expression to block this feedback mechanism. For the first time, it was shown that Sp1 was regulated by itself and this auto-regulation can be interrupted by mithramycin. These findings provide a novel paradigm of synergism between anti-angiogenic and chemotherapeutic reagent. This might help physician in designing regimens that can improve anti-angiogenic activity and reverse resistance in anti-angiogenic therapy.

## DISCUSSION

In this chapter, it was demonstrated that mithramycin, a Sp1 inhibitor, has significant anti-pancreatic cancer capability. The experimental results indicated that mithramycin has strong anti-angiogenic capability with no systemic side effects observed at a given dose. Low dose of mithramycin treatment did not inhibit tumor angiogenesis significantly when comparing with high dose treatment. But low dose of mithramycin treatment did reduce Sp1 and its downstream angiogenic molecules' expression in tumors. The impact of mithramycin treatment on the expression of Sp1 in xenograft tumors and non-tumor tissues were evaluated. This is to determine the Sp1 inhibition ability of mithramycin on normal tissue at the given dose, which was then used to modify the responses and optimize its treatment strategy. Whether mithramycin preferentially affects neo-angiogenesis and tumor-associated stem cells is unknown and could be further investigated. Besides the anti-angiogenic effect, the mithramycin treatment may produce antitumor activity through a mechanism of induction of tumor cell apoptosis *in vivo*. These indicated that mithramycin may affect tumor cell survival by more than only one anti-angiogenic mechanism. As it was reported by other labs that mithramycin could sensitize tumor cells to apoptosis induction through tumor necrosis factor- $\alpha$ -related apoptosis-inducing ligand, Fas ligand and tumor necrosis factor [177, 179, 180].

Lots of experimental results demonstrated that angiogenesis had important roles in maintained growth and metastasis of pancreatic cancer. Studies reported by different groups showed that pancreatic xenograft tumors in mouse model were suppressed by anti-angiogenic treatment [124, 126, 150]. Several strategies that target VEGF signaling and function have been designed and tested. Pancreatic cancer angiogenesis, growth, and metastasis were inhibited and survival was improved in nude mouse models. Anti-VEGF antibodies, VEGF antisense oligonucleotides, VEGF-directed ribozymes and VEGF fused to a diphtheria toxin were all demonstrated to be effective. Also VEGF receptor interference was developed, such as dominant-negative flk-1, and the small molecules tyrosine kinase inhibitors targeting VEGF receptors [144, 146, 186, 187]. Even the specific cyclooxygenase-2 inhibitor celecoxib was shown to suppress pancreatic cancer growth and metastasis through inhibiting VEGF expression. The other antioxidant isoflavone genistein was also reported to suppress angiogenesis through down-regulating VEGF [130, 188, 189]. It was also reported that the expression of VEGF correlates with MVD status and disease progression in different types of tumors [58, 84]. All the results from different clinical researches and experimental designs using different approaches indicated that angiogenesis is very importance in tumor biology. VEGF played a crucial role in tumor angiogenesis.

In xenograft mouse models, it has been shown that targeting VEGF produced inhibited tumor growth, but the VEGF targeting reagent was reported to have limited response when used as mono-agent regimen in clinic studies [148, 190].

Tumor cells need to be destroyed instead of to be kept static by therapeutic drugs to cure cancer. It has been shown that anti-angiogenic approaches produced more effective results when they were used together with chemotherapy and/or radiation therapy. In combination with other strategies VEGF targeting were reported to be more effective for neuroendocrine tumor, renal cell carcinoma, and some sarcomas [191-196]. Also, gemcitabine was reported to facilitate anti-angiogenic therapies when used together with anti-VEGF, anti-VEGF receptor and anti-EGFR antibodies [185, 197-199]. Anti-angiogenic therapies that target single molecule produced limited therapeutic effect suggested the importance of targeting several angiogenic signals and effectors factors at the same time [58]. Targeting these individual molecules might also potentially result in resistance to the targeting drug through feedback mechanisms.

Recently Bevacizumab was used in combination with gemcitabine, capecitabine for advanced local-regional pancreatic cancer in randomized phase II and III clinical trials. It was also used as combination with rapamycin, everolimus, erlotinib or oxaliplatin in treatments of other type of cancer and produced promising results in clinical trials [200-202]. Mechanisms have been proposed for the synergy between anti-angiogenic therapy and chemotherapy. It was proposed that VEGF targeted therapeutics may stabilize mature blood vascular since VEGF was demonstrated to be a vascular permeability factor. Thus interstitial fluid pressure was decreased, which may in turn enhance chemotherapy reagent

delivery [203]. There is research even pointed out that pancreatic cancer treatment needs to enhance vasculature to achieve better chemotherapeutic results [204]. On the other hand, it was also proposed that Bevacizumab treatment may reverse apoptotic resistance mediated by neuropilin receptors [124, 205].

Several other angiogenic growth factors, such as FGFs, EGF, HGF, transforming growth factor- $\alpha$  and platelet-derived growth factor were also reported to be over-expressed in pancreatic cancer cells [58, 84]. It has been reported that the expression of these and many other factors correlate with increased vasculature and poor prognosis in pancreatic cancer patients [58, 84, 85]. Therefore, other factors are involved in the pancreatic cancer growth and metastasis process as well as VEGF. In autocrine and paracrine fashion, these factors produce angiogenic activity to promote pancreatic cancer cell growth and angiogenesis and enhance pancreatic tumor metastasis eventually [84, 85]. It was reported that angiogenesis and growth of pancreatic cancer could be interrupted by interfering the expression or function of the angiogenic factors discussed in mouse models. Such as dominant-negative IGF-1R (IGF/IGF-1R interferer) or NK4 (a competitive antagonist of HGF) treatment produced anti-angiogenic activity [58, 84]. It would be predicted that targeting these angiogenic factors at the same time would produce promising therapeutic effect. However, it has been a clinical challenge for multiple targeting at the same time.

Previous experimental results have indicated that a potential underlying mechanism for over-expression of various angiogenic factors existed. With this mechanism, angiogenesis was collectively regulated with the over-expression of the angiogenic factors as the directed result [58, 88]. Angiogenic signals carried by these angiogenic factors transduced through intra-cellular signal network and converged to transcription factors. In pancreatic cancer one of the transcription factor Sp1 is reported to be constitutively over-expressed *in vitro*. The constitutive and inducible VEGF expression depends on the Sp1 constitutive activity [58, 61, 88, 108, 134]. It was also reported that over-expression of Sp1 is correlated with MVD in human gastric cancer specimen assessed by immunohistochemistry staining [58, 88]. In the present study mithramycin treatment was shown to interpose Sp1 recruitment onto its own promoter, which blocked Sp1 transcription and decreased Sp1 protein. This is correlated with the suppressed tumor angiogenesis and growth *in vivo*. All of these evidences indicate that Sp1 plays important roles in the regulation of angiogenesis and Sp1/VEGF signaling pathway is important in pancreatic cancer.

Mithramycin treatment at the ineffective low dose did not produce significant antitumor activity in the present study. But in tumors the VEGF protein level was reduced when comparing with control group. This result indicated that mithramycin treatment at the given dose inhibits Sp1 and VEGF expression, but there are small amount of extracellular matrix-associated VEGF in the tumor bed or the circulating VEGF remaining. These two parts of VEGF residues may

initiate angiogenesis. The angiogenic phenotype may also be maintained by the small amount of VEGF leftover. It is critical, if that is the case, to remove the remaining VEGF to diminish the angiogenic signals. This might be the reason that why the given low dose of mithramycin treatment cannot produce significant antitumor activity. It was also observed that many Sp1 downstream angiogenic molecules, including VEGF, were up-regulated by low dose of Bevacizumab treatment. This might be the reason why treatment with Bevacizumab as single drug was not sufficient to produce sustained anti-angiogenesis. However, the combination of Bevacizumab and mithramycin can neutralize soluble and membrane bounded VEGF, block the feedback on Sp1 and its downstream angiogenic molecules and produce synergistic anti-angiogenic effect in pancreatic xenograft mouse models. All of these experimental results suggesting that Sp1 and Sp1/VEGF signaling are very important in pancreatic cancer angiogenesis.

Collectively, the present experimental results suggest that VEGF targeting treatment, such as Bevacizumab, may trigger the drug resistance in pancreatic cancer. The positive feedback through activated Sp1 expression and Sp1 downstream angiogenic molecules' activation as the consequence might be one of the underlying mechanisms. The combination treatment of mithramycin and Bevacizumab can block Sp1 transcriptional regulation and neutralize VEGF, which is a novel strategy of targeting angiogenesis. The proposed molecular drug resistance formation model might be extended to other type of cancer

therapeutics besides pancreatic cancer and other type of therapy besides anti-angiogenesis. The rationale represented by the combination use of Bevacizumab and mithramycin would push the pancreatic cancer anti-angiogenic therapy step forward.



## **CHAPTER III**

### **Synergistic Antitumor Regulation of Mithramycin and Tolfenamic acid of Pancreatic Cancer in Mouse Models**

#### **INTRODUCTION**

##### **ANTI-ANGIOGENIC THERAPY APPROACHES AND REATIONAL**

As it has been discussed previously, angiogenesis is an important process for tumor cell survival and metastasize to distant organs. It is dynamically regulated by lots of angiogenic factors and angiogenic inhibitors [66, 181]. Under the hypoxia condition in tumor micro-environment, angiogenic factors are over-expressed and predominate over angiogenic inhibitors. These angiogenic factors whose over-expression leads to the imbalance are the targets of current anti-angiogenic targeted therapies [66, 82, 86, 87, 206]. Among the growing list of angiogenic factors, VEGF is considered as one of the very important ones for most of the tumor types.

Among current anti-VEGF target therapies, Bevacizumab is considered to be one of the most successful ones. Bevacizumab is the first FDA approved anti-angiogenic medicine. It is a humanized murine monoclonal Antibody [207]. Its murine origin is A4.6.1. It recognizes VEGF<sub>121</sub>, 165 and 189 [208]. The *in vivo* embryonic chicken angiogenesis and vascular permeability assay showed it can

neutralize VEGF. In preclinical study, xenograft tumor growth was inhibited by Bevacizumab treatment [148, 183, 209-211]. Bevacizumab is now used in different phases for different types of cancer. It is now the 1st line medicine of colon cancer treatment.

It is known that VEGF transcriptional control is also being used. The most important one is HIF-1 $\alpha$  targeted therapy from each level. HIF-1 $\alpha$  is monomer that can form heterodimeric transcription factor HIF. HIF-1 $\beta$  (or Arnt) is the other component. HIF can bind to hypoxia-response element (HRE, which contains the consensus core sequence 5'-R(A/G)CGTG-3' ) and activates the transcription of VEGF and VEGFR1 [212]. HIF-1 $\alpha$  is over-expressed in most types of solid tumors. Its expression might be activated by intra-tumoral hypoxia and genetic alterations, including oncogenic gain-of-function mutations and tumor-suppressor loss-of-function mutations, such as VHL (Von Hippel-Lindau) and PTEN. Under hypoxic condition, VEGF transcription is up-regulated by HIF heterodimer binding on the HRE in its promoter region. There is also an internal ribosomal entry site allowing preserved translation when facing normal cellular hypoxic shutdown. VEGF biological function is also influenced by hypoxia-inducible expression and post-transcriptional regulation of VEGF receptors. Mouse genetics study shows inactivation of HIF-1 $\alpha$  resulting in abnormal vascular development and embryonic lethality. The HIF-1 $\alpha$ <sup>-/-</sup> mice have angiogenic defects in both the yolk sac and the development of embryonic tissue [213].

A variety of genetic and pharmacologic approaches have been used to target HIF-1 $\alpha$ . As a transcription factor, its DNA binding affinity can be targeted; its protein stability can also be targeted since it has a natural stable/degraded regulation mechanism; its mRNA stability can also be targeted using siRNA (small interfering RNA) technology; its protein level may be targeted by regulating the translation process; the important PI3K/Akt signaling pathway is another aspect of HIF-1 $\alpha$  targeting to affect its transcription; the nuclear translocation of HIF-1 $\alpha$  and the dimerization process may be desirable targets for the inhibition of HIF-1 activity [214].

As a receptor of VEGF signaling pathway, the most important angiogenesis signaling pathway, VEGFRs are certainly the hot targets of anti-angiogenic therapy. Although with questionable specificity, small molecule tyrosine kinase inhibitor (TKI) is always the hot spot of VEGFR targeting research. VEGFR can also be targeted by chimeric receptor and VEGF trap (soluble chimeric VEGFR). They can all inhibit tumor growth *in vivo*. Antisense oligonucleotides is also used to target VEGFR. Besides these, small molecule VEGFR-2 kinase and phosphorylation inhibitors are used. Neutralizing Antibodies directed against VEGFR such as DC101, IMC-1C11 and CDP791 were also developed.

## **USE OF MITHRAMYCIN IN SP1 MANIPULATION**

Mithramycin is an antibiotic that has antitumor activity as shown in the last chapter. It is produced by the bacteria of the genus *Streptomyces sp* and can

bind to Sp1 site in DNA sequence [164, 165]. Mithramycin can compete with transcription factor binding to promoters and block transcription activation. After binding on DNA, it forms a stoichiometry drug-metal complex through the chelation with divalent ion. This interaction is non-covalent and is reversible [168, 175, 215]. It is believed that mithramycin selectively competing with Sp1 to regulate the transcription of genes that have Sp1 site(s) in their promoter region [175, 176, 216]. As it has been shown in last chapter, mithramycin treatment down-regulates Sp1 protein through blocking Sp1 transcriptional auto-regulation and decreases Sp1 downstream angiogenic molecules as a consequence. The major mechanism of mithramycin antitumor effect is believed to inhibit Sp1 activity [217]. While this manipulation to Sp1 protein is the result of Sp1mRNA transcription blockage instead of working on the protein molecules directly.

### **TOLFENAMIC ACID (TA) HAS ANTI-TUMOR ACTIVITY**

Tolfenamic acid is a prostaglandin as well as leukotriene synthesis inhibitor through blocking cyclooxygenase catalytic activity. It is a well-documented and effective non-steroidal anti-inflammatory drug for treatment of migraine symptom. It can relieve endometriosis symptoms in patients with pelvic endometriosis through inhibiting prostaglandin synthetase and antagonizing prostaglandins at the target level [218]. It is also used to diminish the local reactions after telecobalt therapy [219]. TA has a long history to be used in treating acute migraine attack either alone or with caffeine, metoclopramide and pyridoxine as adjuncts [220-

222]. When TA was used together with sumatriptan, migraine recurrence decreased dramatically [223]. Recently, TA is reported to be an urokinase plasminogen activator inhibitors in a computational study by docking nutraceuticals to the 3D structure of urokinase [224]. It was also reported to activate Sp1, Sp3, and Sp4 protein degradation in pancreatic cancer cells and this was accompanied by decreased VEGF and VEGF receptor 1 in both mRNA and protein level [225, 226].

In this chapter, the synergistic anti-tumor effect of mithramycin and TA combination and the corresponding anti-angiogenic, cell growth inhibition mechanism were investigated.

## **MATERIALS AND METHODS**

### **Chemicals and Reagents Information**

Mithramycin (1 mg/vial crystal powder) and TA were purchased from Sigma Chemical Co. (St. Louis, MO). Mithramycin was dissolved in sterile water diluted for actual use. TA was mixed with corn oil (Sigma Chemical Co., St. Louis, MO) to corresponding concentration. For animal experiments, mithramycin was administered by intraperitoneal injection twice a week or as indicated otherwise. TA was administered through oral gavage.

### **Cell Lines and Culture Conditions Information**

The resources of human pancreatic adenocarcinoma cell lines PanC-1, FG and BxPC3 are the same as previous chapters. The cell lines were maintained in plastic flasks as adherent monolayers in minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and a vitamin solution (Flow Laboratories, Rockville, MD).

### **Animals Information**

Female athymic BALB/c nude mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used when they were 8 weeks old.

The animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and National Institutes of Health in M D Anderson Cancer Center animal facility.

### **Western Blot Analysis Protocol**

Whole-cell lysates were prepared from human pancreatic cancer cell lines or tissues [61]. Standard Western blotting was performed using polyclonal rabbit antibodies against human and mouse Sp1, VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) and the anti-rabbit IgG antibody, a horseradish peroxidase-linked F(ab')<sub>2</sub> fragment obtained from a donkey (Amersham, Arlington Heights, IL). Equal protein-sample loading was monitored by probing the same membrane filter with antibodies against glyceraldehyde-3-phosphate dehydrogenase [227]. The probe proteins were detected using the Amersham enhanced chemiluminescence system according to the manufacturer's instructions.

### **Matrigel Plug Assay**

Matrigel (200µl) containing  $2 \times 10^6$  cells were injected subcutaneously into nude mice (2 injection sites per mouse). The Matrigel plugs were recovered from the mice 10 days after injection and carefully stripped of host tissues. After

photomicrography, the Matrigel plugs were weighed and homogenized in 1 ml of distilled water and then centrifuged at 10,000 revolutions per minute for 5 minutes. The supernatants were collected for hemoglobin measurement using Drabkin solution (Sigma Chemical Company, St. Louis, MO) and a Microplate Manager enzyme-linked immunosorbent assay reader at 540 nm according to the manufacturer's instructions. The relative hemoglobin concentrations were calculated and further normalized according to the weights of the plugs.

### **Immunohistochemical Analysis and Quantification of Tumor MVD**

For CD31 staining, frozen tissue sections (5 $\mu$ m thick) were fixed in acetone. Endogenous peroxidase in the specimens was blocked using 3% hydrogen peroxide in PBS for 12 minutes. The specimens were incubated for 20 minutes at room temperature in a protein-blocking solution consisting of PBS (pH 7.5) containing 5% normal horse serum and 1% normal goat serum and then incubated overnight at 4°C in a 1:100 dilution of monoclonal goat anti-CD31 (PECAM1-M20), polyclonal rabbit anti-Sp1, polyclonal rabbit anti-VEGF or polyclonal rabbit anti-PCNA antibodies (Santa Cruz Biotechnology) [229]. The specimens were then rinsed and incubated with peroxidase-conjugated anti-goat or anti-rabbit IgG for 1 hour at room temperature. Next, the slides were rinsed with PBS and incubated with diaminobenzidine (Research Genetics, Huntsville, AL) for 5 minutes. Frozen sections of the specimens were then washed three times with distilled water, counterstained with Mayer's



hematoxylin (Biogenex Laboratories, San Ramon, CA), and washed once each with distilled water and PBS. The slides were mounted with Universal Mount (Research Genetics) and examined under a bright-field microscope. A positive reaction was indicated by a reddish-brown precipitate in the cytoplasm (CD31) or nuclei (Sp1). For quantification of tumor MVD, vessels on each section were counted in five high-power fields (magnification, x200 [x20 objective and x10 ocular]) as described previously [230, 231].

### **Chromatin Immunoprecipitation Protocol**

Chromatin was prepared from cells and tumors as described previously [59]. Chromatin Immunoprecipitation (ChIP) assay was done using the Chromatin Immunoprecipitation Assay Kit (Upstate Cell Signaling Solutions) according to the manufacturer's instructions. Briefly, DNA cross-binding proteins were cross-linked with DNA and lysed in SDS lysis buffer. The lysate was sonicated to shear DNA to 200 to 1000 bp. After pre-clearing with a salmon sperm DNA/protein A agarose – 50% slurry for 30 min at 4°C, chromatin samples were immunoprecipitated overnight with no antibody or an anti-Sp1 antibody (PEP2). The region between -224 and -53 bp of the Sp1 promoter was amplified using the following primers: sense, 5'-caggcacgcaacttagtc-3', and antisense, 5'-gtaaggaggaggagcag-3'. The region between -272 and +18 bp of the VEGF promoter was amplified using the following primers: sense, 5'-ccgcgggcgcgtgtctctgg-3', and antisense, 5'-tgccccaagcctccgcgatcctc-3'. PCR

products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

### **Statistical Analysis**

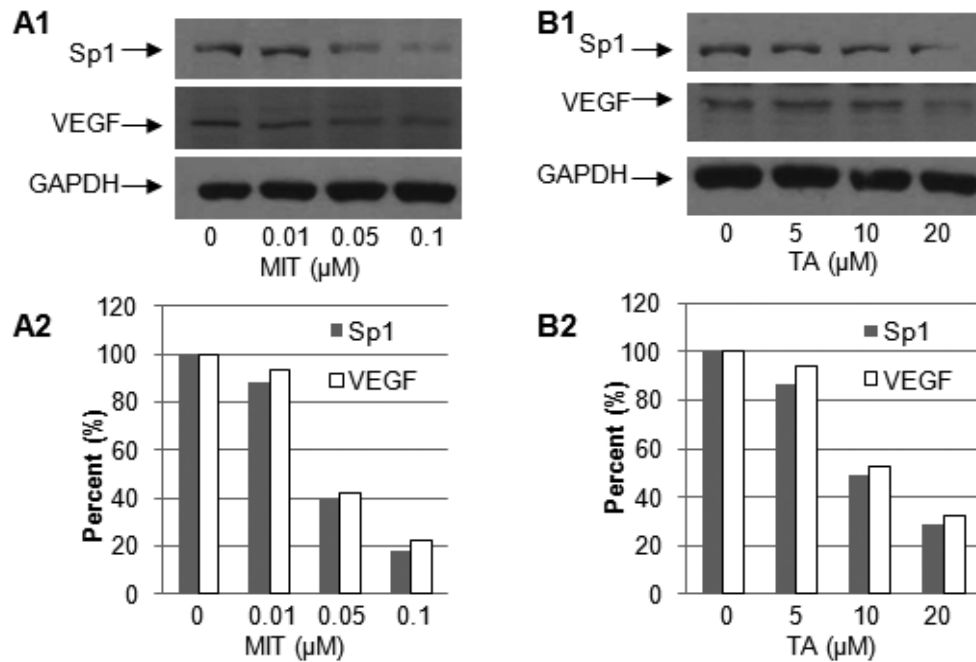
Each experiment was done independently at least twice with similar results; one representative experiment is presented. The significance of the *in vitro* data was determined using Student's *t* test (two-tailed), whereas the significance of the *in vivo* data was determined using the two-tailed Mann-Whitney *U* test. For the *in vivo* experiments, the overall survival duration was calculated using the Kaplan-Meier method. The log-rank test was used to compare the survival duration between groups.  $P \leq 0.05$  was deemed significant.

## RESULTS

### **Effects of treatment with mithramycin and/or tolfenamic acid on the gene expression in human pancreatic cancer cells**

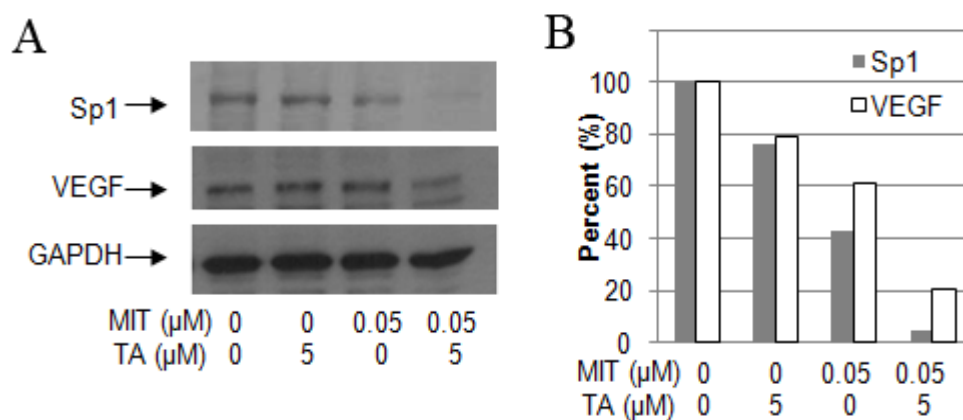
To determine the impacts of mithramycin and TA treatment on gene expression in pancreatic cancer cells, PANC-1 and BxPC3 cells were incubated in a medium alone or a medium containing mithramycin (0.01, 0.05 and 0.1  $\mu\text{mol/L}$ ) and/or TA (5, 10, 20  $\mu\text{mol/L}$ ). Western blot results showed that Sp1 protein level was down-regulated by both mithramycin and TA treatment as single reagent in a dose-dependent pattern after 24 hours of treatment (Fig. 24A and 24B) *in vitro*.

As shown in Fig. 25, low dose of mithramycin and TA combination treatment can significantly down-regulate Sp1 protein level. These data suggested that high dose of mithramycin and TA treatment can decrease Sp1 protein level significantly at around 24 hours and low dose of mithramycin and TA combination produced synergistic down-regulation effect on Sp1 protein at around 12 hours.



**Figure 24 Tolifenamic acid, mithramycin treatments down-regulate Sp1 *in vitro***

PANC-1 cells were incubated in medium alone or a medium containing mithramycin or TA. Total protein lysates were harvested from the cell cultures, and the level of Sp1 and VEGF protein expression was determined using Western blot analysis. Equal protein-sample loading was monitored by probing the same membrane filter with an anti-GAPDH antibody. A1, mithramycin (0.01, 0.05 and 0.1 μmol/L) were used to treat PANC-1 cells and samples were harvested at 24hours. A2, the quantitative results of Sp1 and VEGF in A1 normalized with GAPDH; Blank treatment was set as 100%. B1, TA (5, 10, 20 μmol/L) was used to treat PANC-1 cells and sample were harvested at 24 hours. B2, the quantitative results of Sp1 and VEGF in B1 normalized with GAPDH; Blank treatment was set as 100%.

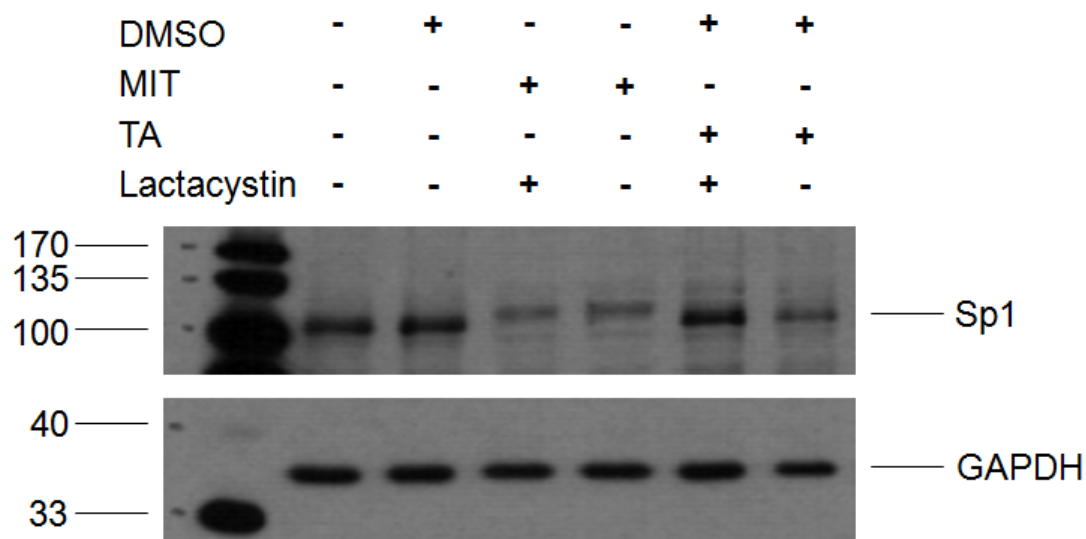


**Figure 25 Tolfenamic acid/mithramycin treatments down-regulate Sp1 *in vitro***

PANC-1 cells were incubated in medium alone or a medium containing mithramycin and/or TA. Total protein lysates were harvested from the cell cultures, and the level of Sp1 and VEGF protein expression was determined using Western blot analysis. Equal protein-sample loading was monitored by probing the same membrane filter with an anti-GAPDH antibody. A, Mithramycin (0.05  $\mu$ mol/L) and TA (5  $\mu$ mol/L) was used to treat PANC-1 cells and samples were harvested at 12 hours. B, the quantitative results of Sp1 and VEGF in A normalized with GAPDH. Blank treatment was set as 100%.

## **Tolfenamic acid and mithramycin treatments down-regulate Sp1 protein through different mechanism**

As it was shown in last chapter, mithramycin down-regulates Sp1 expression through interfere its recruitment on its own promoter to block Sp1 mRNA transcription. To determine how TA treatment regulates Sp1 protein expression in pancreatic cancer cells, PANC-1 cells were incubated in a medium alone or a medium containing mithramycin (0.1  $\mu\text{mol/L}$ ) with or without lactacystin (2  $\mu\text{mol/L}$ ) or TA (20  $\mu\text{mol/L}$ ) with or without lactacystin (2  $\mu\text{mol/L}$ ) for 24 hours. Lactacystin is a selective inhibitor of the proteasome. Cells that were treated with 0.1% DMSO was set as control to TA treatment. Western blot results showed that Sp1 protein level was down-regulated by both mithramycin and TA treatment without lactacystin. However, when cells were treated with both TA and lactacystin, Sp1 protein level was not decreased. Thus, TA appears to activate proteasome-dependent Sp1 degradation, while mithramycin does not affect proteasome-dependent Sp1 degradation because the combination of mithramycin and lactacystin down-regulated Sp1 protein to the same level as mithramycin alone. This result shows that both mithramycin and TA down-regulate Sp1 protein expression but through different mechanism.



**Figure 26 Through different mechanism tolfenamic acid mithramycin treatments down-regulate Sp1 *in vitro*** PANC-1 cells were incubated in medium alone or a medium containing DMSO (0.1%), mithramycin (0.1  $\mu\text{mol/L}$ ), TA (20  $\mu\text{mol/L}$ ) or lactacystin (2  $\mu\text{mol/L}$ ) as shown. Total protein lysates were harvested from the cell cultures, and the level of Sp1 protein expression was determined using Western blot analysis. Equal protein-sample loading was monitored by probing the same membrane filter with an anti- GAPDH antibody.

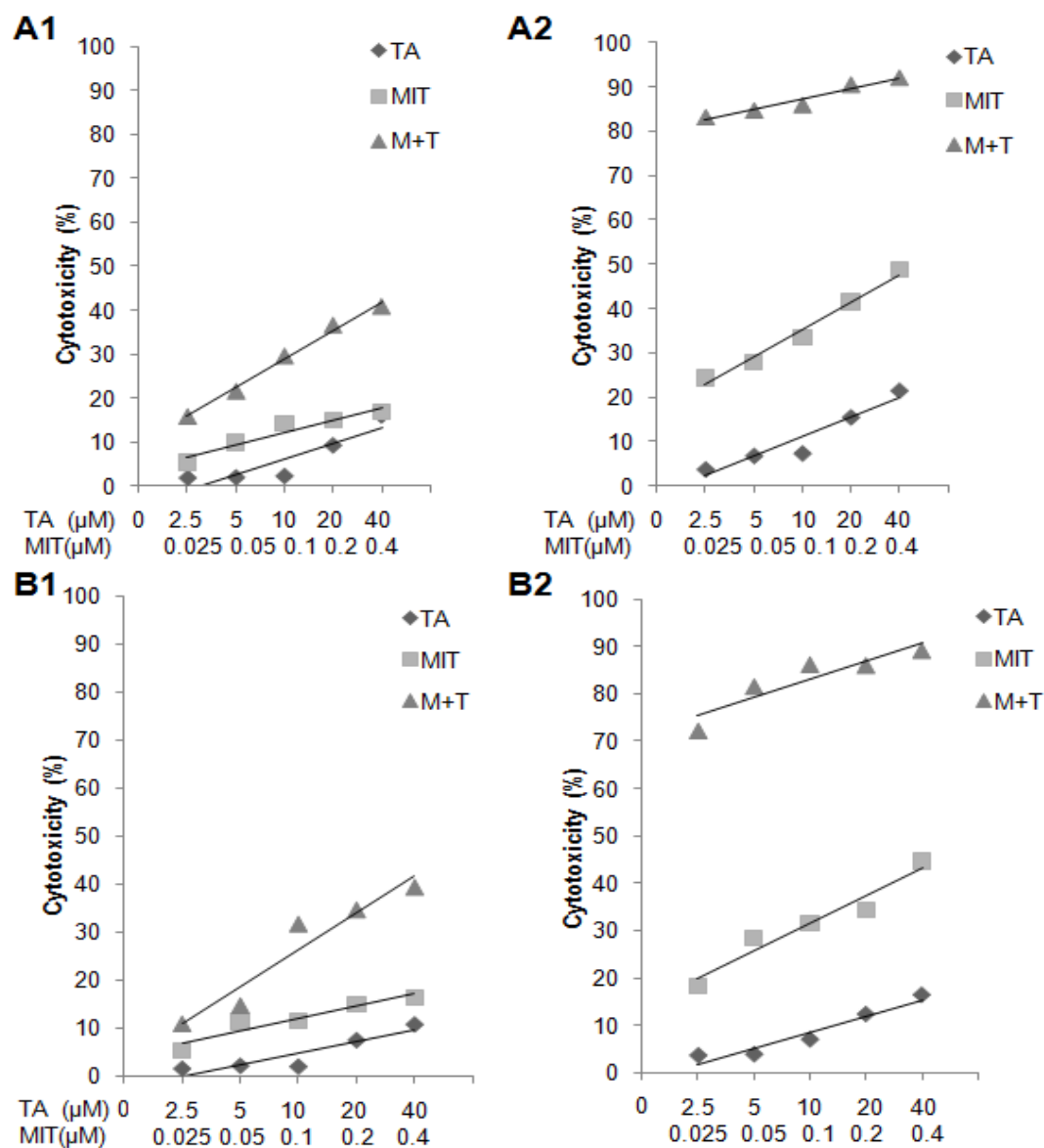
### **Synergistic cytotoxicity of mithramycin and tolfenamic acid in human pancreatic cell lines *in vitro***

MTT assay was performed to determine the pancreatic cancer cell killing effect *in vitro*. FG and BxPC3 cells were treated with mithramycin (0.025, 0.05, 0.1, 0.2, 0.4  $\mu\text{mol/L}$ ) and/or TA (2.5, 5, 10, 20, 40  $\mu\text{mol/L}$ ) for 24 to 48 hours. Figure 27 shows the MTT results. Drug concentration was optimized to the extent in which there is no extensive cytotoxic effect by a single drug treatment. Under the condition, mithramycin and TA showed higher cell killing effect in combination treatment.

To determine the type of mithramycin and TA treatment interaction effect, the MTT cell experimental data were subjected to further statistical analysis. Loewe additivity model, one of the general reference models for evaluating drug interactions, was used [232]. Based on this model, S-PLUS/R was used to evaluate mithramycin and TA interaction. Basically Chou and Talalay's median effect equation was used to do the calculation [233]. FG (Fig 28) and BxPC3 (Fig 29) analyses output were shown. Table 1 shows the estimated interaction indices from the corresponding fitted dose-effect curve (Fig. 28 A2 and B2) respectively. Table 2 shows the estimated interaction indices from the corresponding fitted dose-effect curve (Fig. 29 A2 and B2) respectively. The interaction was considered to be synergy if the interaction index is  $< 1$ , while was considered to be additivity when interaction index is equal to 1 [232]. When the standard

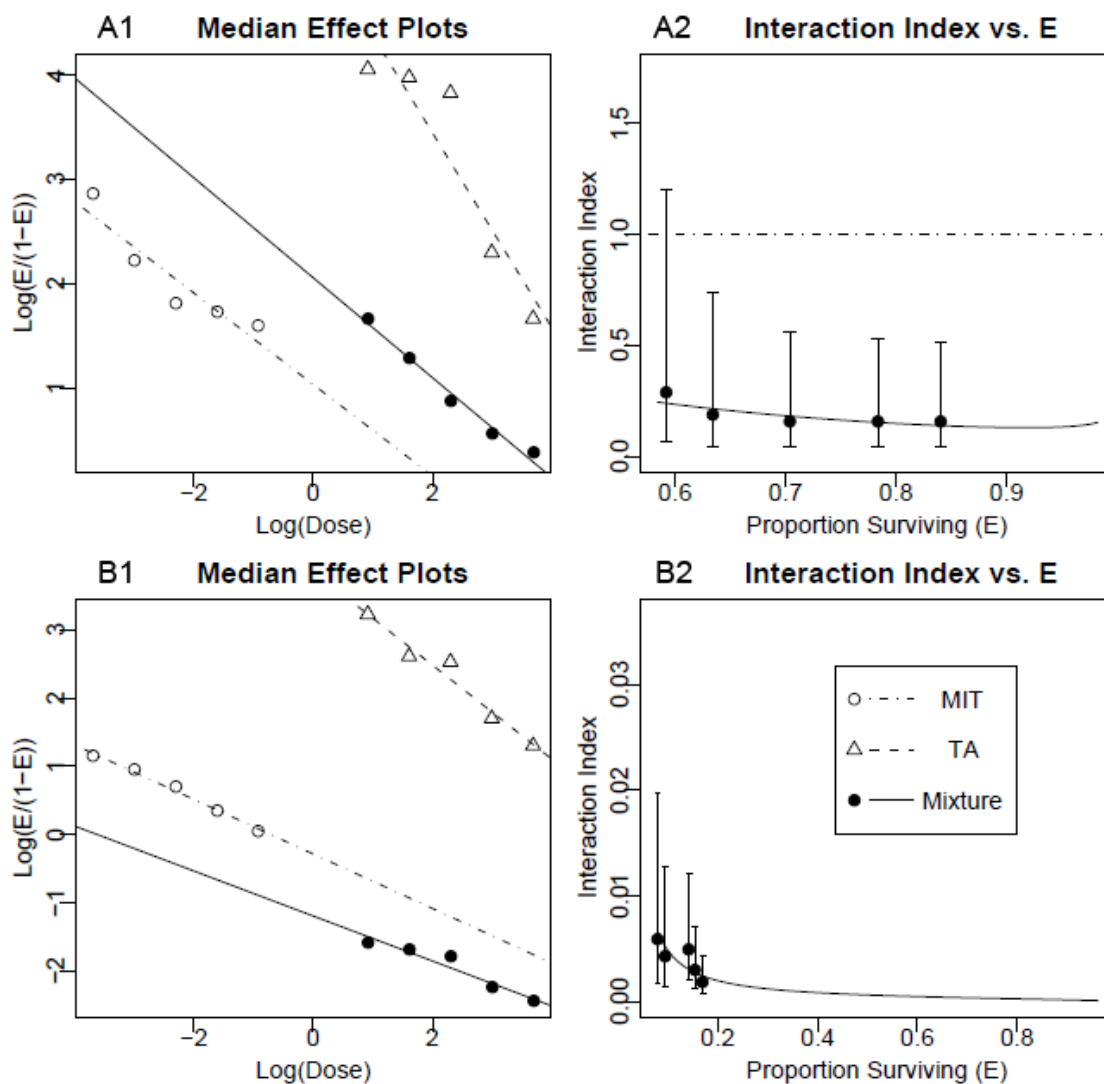


deviation is span 1, the interaction was also considered to be additivity for restriction analysis. All of the calculated interaction indexes were less than 1. But in Fig. 28, 4 of the 5 of 24 hours and all 48 hours data points and in Fig. 29, 3 of the 5 of 24 hours and all 48 hours data points that were considered to be synergy for restriction analysis. This experimental result indicated that the combination of mithramycin and TA produced synergistic cytotoxicity on FG and BxPC3 cells *in vitro*.

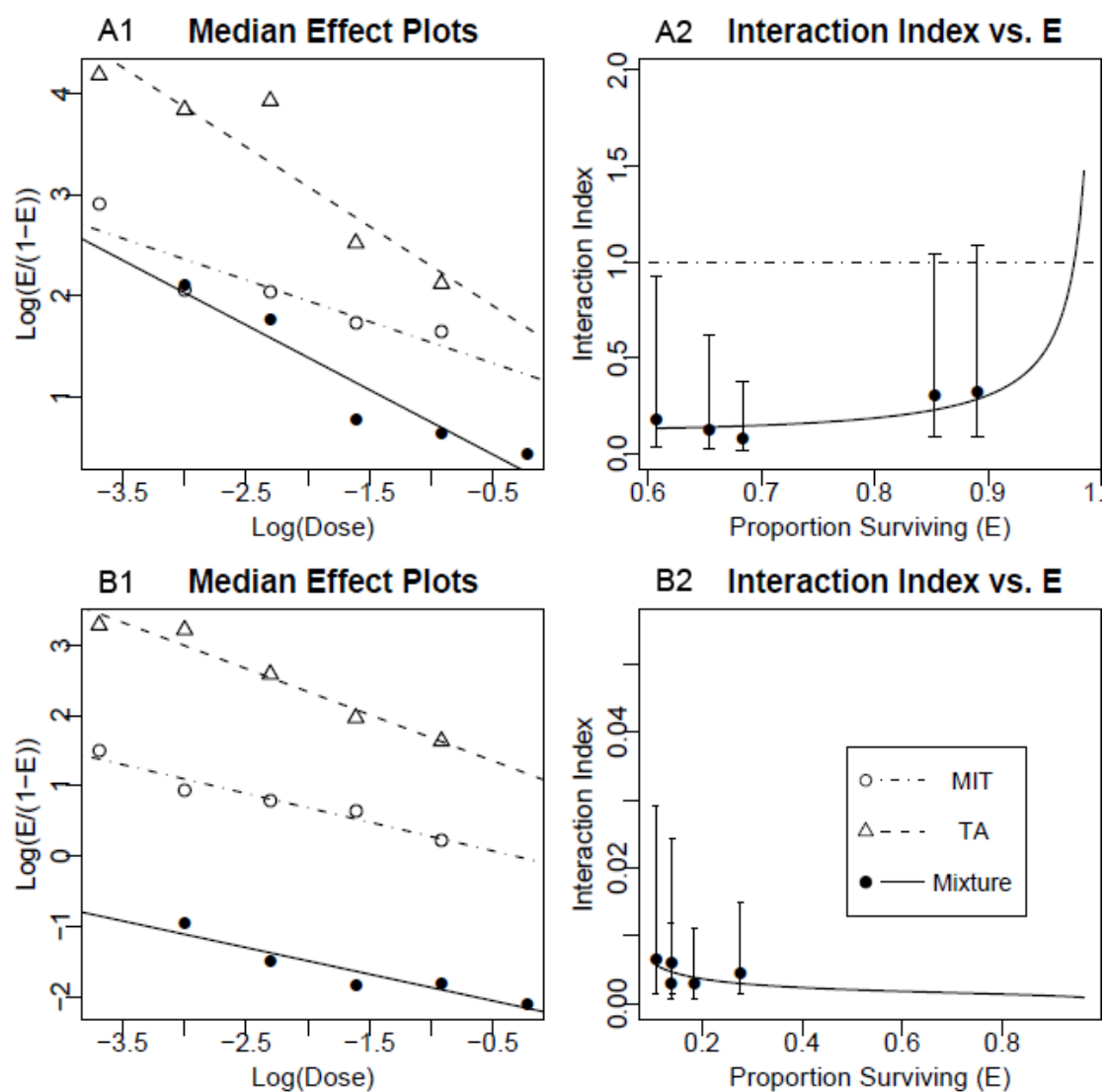


**Figure 27** Mithramycin and tolfenamic acid treatment inhibit cell growth *in vitro* FG

(A) and BxPC3 (B) cells were treated with mithramycin at concentrations ranging from 0.025 to 0.4 μmol/L and TA from 2.5 to 40 μmol/L for 24 (A1, B1) and 48 hrs (A2, B2). Cytotoxicity was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay.



**Figure 28 Synergistic cytotoxicity of mithramycin and tolafenamic acid *in vitro* in FG cell** MTT assay data of FG cell in Figure 26 was analyzed with S-PLUS/R software using Chou and Talalay's median effect equation. A, 24 hours data. B, 48 hours data.



**Figure 29 Synergistic cytotoxicity of mithramycin and tolfenamic acid *in vitro* in BxPC3 cell** MTT assay data of BxPC3 cell in Figure 26 was analyzed with S-PLUS/R software using Chou and Talalay's median effect equation. A, 24 hours data. B, 48 hours data.

**Table 1** Statistics of interaction of mithramycin and tolfenamic acid in the FG cell cytotoxicity experiment

Time Point	Interaction	Additivity	Estimated	95% confidential	Conclusion
	Mithramycin	TA	Value	interval	
	(nmol/L)	( $\mu$ mol/L)			
24 hours	25	2.5	0.156	[0.047, 0.515]	Synergy
	50	5	0.156	[0.046, 0.531]	Synergy
	100	20	0.133	[0.042, 0.558]	Synergy
	200	20	0.188	[0.048, 0.740]	Synergy
	400	40	0.289	[0.069, 1.205]	Additivity
48 hours	25	2.5	0.0018	[0.0008, 0.0043]	Synergy
	50	5	0.0030	[0.0013, 0.0071]	Synergy
	100	20	0.0050	[0.0020, 0.0122]	Synergy
	200	20	0.0042	[0.0014, 0.0128]	Synergy
	400	40	0.0059	[0.0018, 0.0198]	Synergy

**Table 2** Statistics of interaction of mithramycin and tolfenamic acid in the BxPC3 cell  
cytotoxicity experiment

Time Point	Interaction	Additivity	Estimated	95% confidential	Conclusion
	Mithramycin	TA	Value	interval	
	(nmol/L)	( $\mu$ mol/L)			
24 hours	25	2.5	0.177	[0.093, 1.090]	Additivity
	50	5	0.127	[0.086, 1.043]	Additivity
	100	20	0.081	[0.018, 0.377]	Synergy
	200	20	0.300	[0.026, 0.615]	Synergy
	400	40	0.317	[0.034, 0.921]	Synergy
48 hours	25	2.5	0.0046	[0.0014, 0.0150]	Synergy
	50	5	0.0029	[0.0008, 0.0111]	Synergy
	100	20	0.0029	[0.0007, 0.0118]	Synergy
	200	20	0.0059	[0.0014, 0.0243]	Synergy
	400	40	0.0066	[0.0015, 0.0291]	Synergy

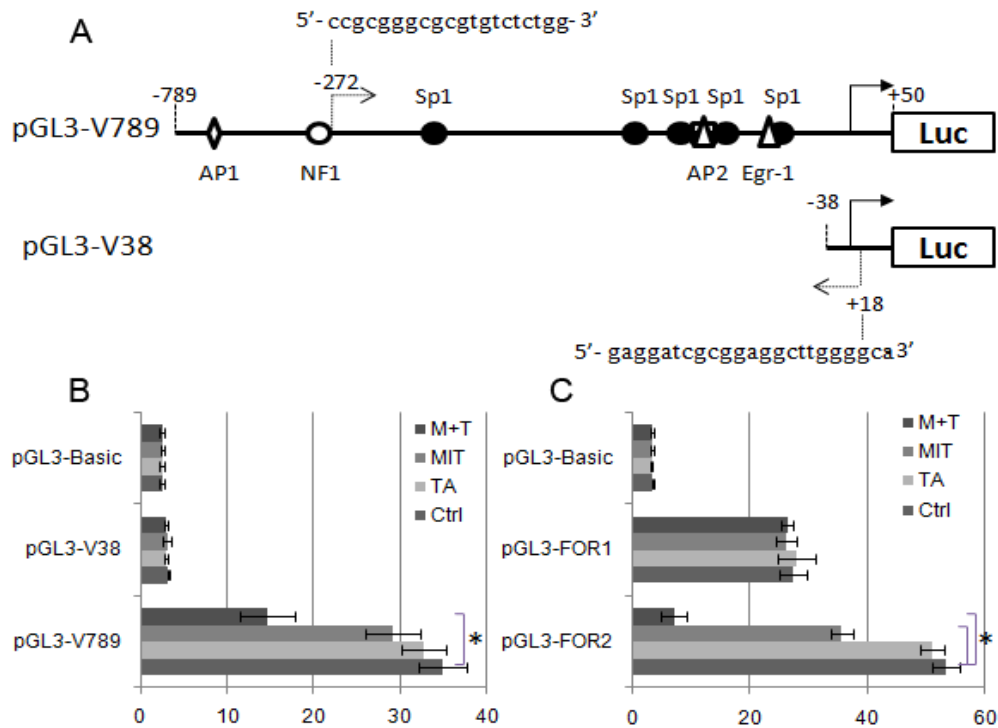
### **Effects of treatment with mithramycin and tolfenamic acid on Sp1 recruitment into the Sp1 and VEGF promoter *in vitro***

In the present experiments, I sought to determine whether treatment with tolfenamic acid and mithramycin regulated Sp1 expression at the transcriptional level. Luciferase assay was performed. Briefly, Sp1 and VEGF (Fig.30A) promoter reporter constructs were transfected into PANC-1 cells and then incubated the cells in 0.01  $\mu\text{mol/L}$  mithramycin or 5  $\mu\text{mol/L}$  tolfenamic acid in culture medium. *In vitro*, treatment with tolfenamic acid did not suppress Sp1 and VEGF promoter activity much, whereas treatment with mithramycin has certain inhibition effect. However, the suppressing ability of mithramycin to Sp1 and VEGF promoter were eliminated by further deletion of Sp1-binding sites in the promoters. Furthermore, combination treatment of mithramycin and TA suppress VEGF (Fig. 30B) and Sp1 (Fig. 30C) promoter activity significantly.

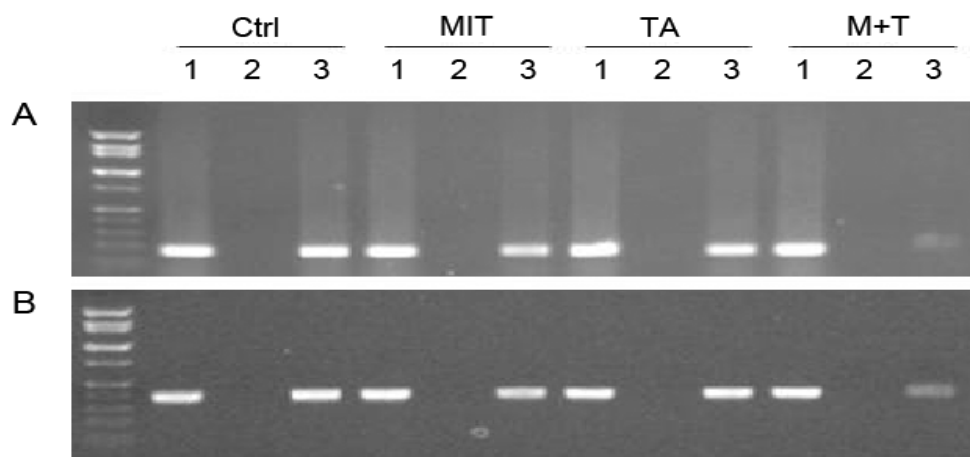
Next ChIP assay was performed as described in Material and Methods. The experimental results were consistent with Luciferase assay results. Mithramycin treatment reduced Sp1 recruitment to its own and VEGF promoter as shown in Figure 31. While treatment with TA has minor affect on Sp1 recruitment to its own and VEGF promoter *in vitro* (Fig. 31). In contrast, treatment with mithramycin and TA combination suppressed Sp1 protein recruitment to its own promoter and VEGF promoter significantly. These data suggested that

mithramycin and TA combination treatment synergistic reduced Sp1 recruitment to its own and VEGF promoter.





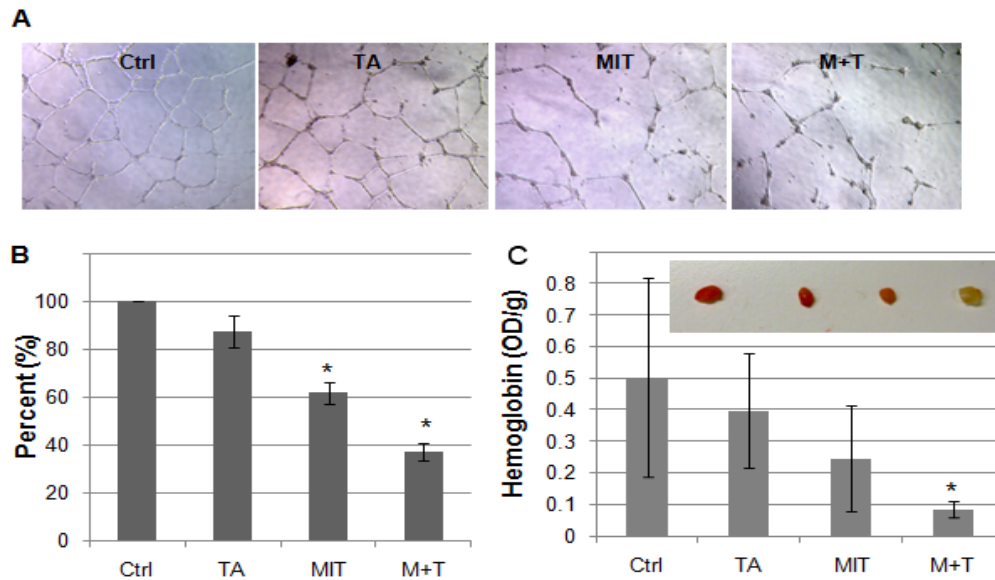
**Figure 30 Effects of tolifenamic acid and mithramycin treatment on Sp1 and VEGF promoter activity *in vitro*.** A, schematic structures of the minimal VEGF promoters. The nucleotide positions and sequences of Sp1-binding sites and PCR forward and reverse primers flanking those sites for ChIP assay are shown. B, VEGF and C, Sp1 promoter reporter constructs were transfected into PANC-1 cells in triplicate and incubated for 12 h. The cells were then incubated for another 24 h in medium alone or a medium containing 0.01 $\mu$ mol/L mithramycin or 5  $\mu$ mol/L TA. Total protein lysates were harvested from the cell cultures for measurement of promoter activity using a luciferase assay kit. The relative promoter activities were assessed, and the activity in treated groups was expressed as the fold change of that in their respective control groups. \*  $P < 0.05$  in a comparison between the treated and respective control groups.



**Figure 31 Effects of mithramycin and tolfenamic acid on Sp1 and VEGF promoter *in vitro*** Chromatin Immunoprecipitation was performed as described in Materials and Methods. Chromatin was extracted from PANC-1 cells that were incubated *in vitro* for 2 d in medium alone or a medium containing 0.5  $\mu\text{mol/l}$  mithramycin, 50  $\mu\text{mol/l}$  TA, 0.5  $\mu\text{mol/L}$  mithramycin + 50  $\mu\text{mol/L}$  TA (M+T). A specific anti-Sp1 antibody and oligonucleotides flanking the (A) Sp1, (B) VEGF promoter regions containing Sp1-binding sites was used. Lane 1, input chromatin DNA; lane 2, chromatin DNA with control IgG; lane 3, chromatin DNA with anti-Sp1 antibody.

### **Anti-angiogenesis effects of mithramycin and tolfenamic acid *in vitro***

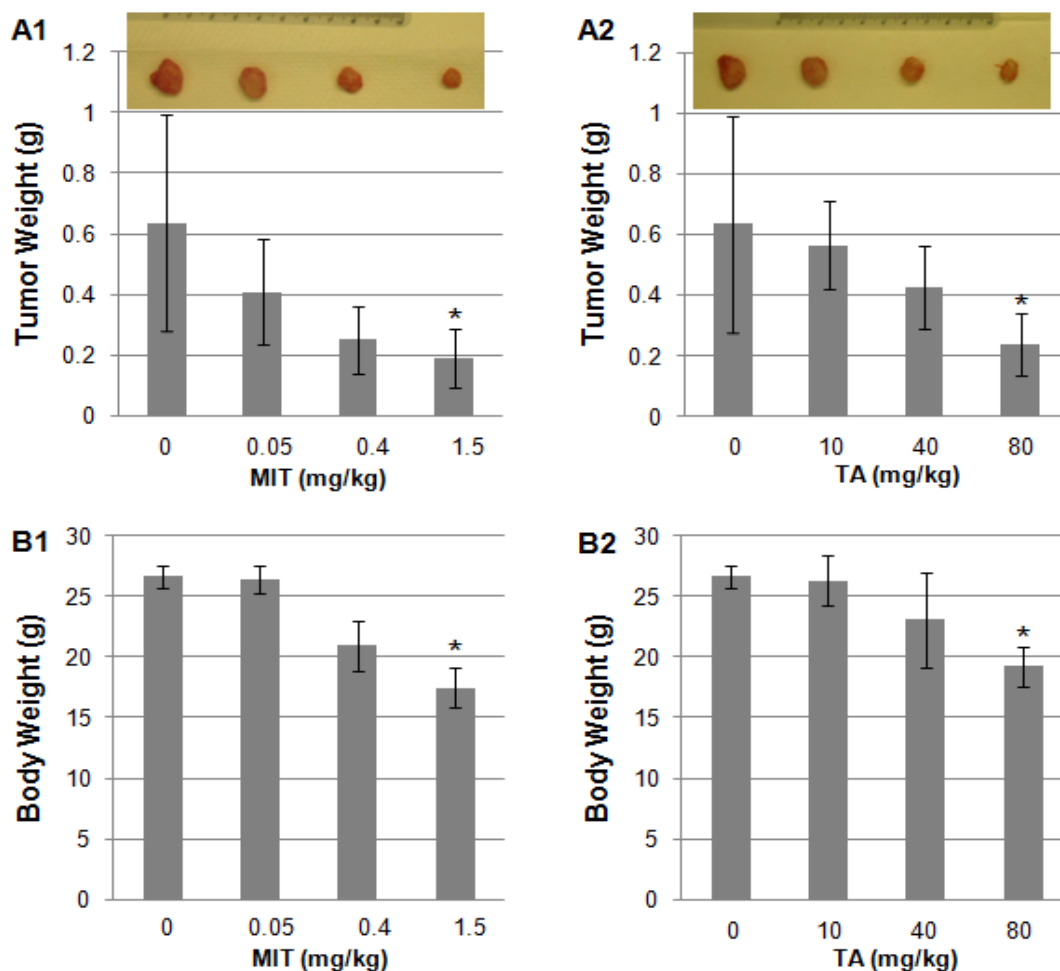
Pancreatic cancer cell PANC-1 was treated with 50  $\mu\text{mol/L}$  tolfenamic acid and/or 0.1  $\mu\text{mol/L}$  mithramycin. Western blot was used to determine Sp1 protein alternation in these cells. Sp1 expression down-regulation was confirmed. Endothelial cell tube formation assay was used to determine the angiogenic potential of the supernatants of PANC-1 culture. The degree of tube formation was assessed as the percentage of cell surface area versus the total surface area (Fig. 32A). Representative photomicrographs were taken *in situ* for tube formation of HUVECs incubated in the supernatants (Fig. 32B). Mithramycin and/or tolfenamic acid treatment reduced the capacity of supernatants of PANC-1 cells to stimulate tube formation of endothelial cells compared with the capacity of supernatants of control PANC-1 cells. The impaired angiogenic potential was confirmed further by *in vivo* Matrigel plug assay (Fig. 32C) (protocol was described in Material and Methods). These data indicated that mithramycin and/or tolfenamic acid treatment reduced the angiogenic potential of PANC-1 cells.



**Figure 32 Mithramycin and tolafenamic acid treatment affect PANC-1 cell angiogenic phenotype** A, Culture supernatants were harvested from PANC-1 cells treated with 0.5  $\mu\text{mol/L}$  mithramycin, 50  $\mu\text{mol/L}$  TA, 0.5 $\mu\text{mol/L}$  mithramycin + 50 $\mu\text{mol/L}$  TA respectively. The angiogenic potentials of the supernatants were determined by an endothelial cell tube formation assay. Representative pictures were taken *in situ* for tube formation in the supernatant of the above 4 groups. (B) The degree of tube formation was assessed as the percentage of cell surface area versus total surface area. Control cell cultures were given arbitrary percentage values of 100. C, For a Matrigel plug assay, Matrigel (200  $\mu\text{l}$ ) that contained  $2 \times 10^6$  PANC-1 cells or PANC-1 cells treated with 0.5  $\mu\text{mol/L}$  mithramycin, 50  $\mu\text{mol/L}$  TA or 0.5  $\mu\text{mol/L}$  mithramycin + 50 $\mu\text{mol/L}$  TA was used as described in the text (see Materials and Methods). It is noteworthy that the down-regulation of Sp1 expression impaired the angiogenic potential of pancreatic cancer cells *in vitro* and *in vivo*. \*  $P < 0.05$  in a comparison between the treated and respective control groups.

### **Antitumor effects of mithramycin and tolfenamic acid in human pancreatic cancer xenograft mouse models**

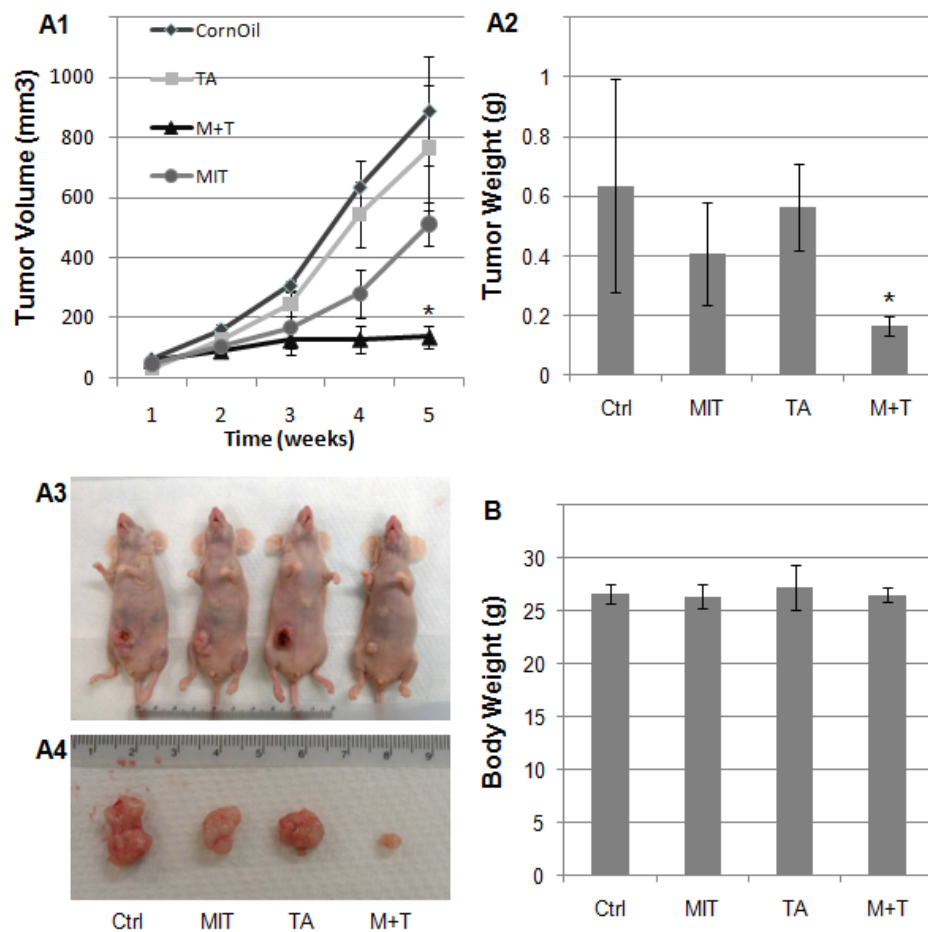
Both mithramycin and TA treatment can down-regulate Sp1, VEGF and VEGFR [225, 226]. However, it is unknown whether these two drugs interact synergistically in regulating Sp1 activity and tumor growth. To determine the two drugs' function on tumor *in vivo*, PANC-1 xenograft tumor mouse model was used. Specifically, dose-response experiments were performed. The animals with PANC-1 xenograft tumor were given different doses of mithramycin (0.1, 0.4, and 1.5 mg/kg; Fig. 33A) via intraperitoneal injection twice a week and TA (10, 40, 80 mg/kg) via oral gavage three times a week. It was found that treatment with mithramycin and TA produced dose-dependent antitumor activity. But the mouse body weight, as a drug cytotoxicity index, also decreased in a dose-dependent manner (Fig. 33B).



**Figure 33 Anti-tumor activity of mithramycin and tolfenamic acid treatment *in vivo***

A-B, Dose-dependent antitumor effects of mithramycin and TA in xenograft models of human pancreatic cancer. A, PANC-1 cells were injected into the subcutis of nude mice (n = 5). When tumors reached around 4 mm in diameter, the animals received different doses of (A1) mithramycin (0.1, 0.4, 1.5mg/kg) via i.p. injection twice a week, (A2) TA (10, 40, 80 mg/kg) via oral gavage three times a week. Tumors were weighted at the time of experiment termination. (B) Mice bodies were also weighted at the same time. Columns, mean weights; bars, SD. \*  $P < 0.05$  in a comparison between the treated and respective control groups.

Next, an experiment using PANC-1 tumor cell mouse models in which PBS, mithramycin (0.1 mg/kg), TA (10 mg/kg), or M+T were administered to a group of nude mice was performed. It was found that administration of TA or mithramycin alone produced minor antitumor activity. In contrast, administration of mithramycin and TA produced significant antitumor activity (Fig. 34A1 and 34A2). Furthermore, the use of low dose of TA and MIT produced synergistic antitumor activity without producing significant systemic side toxicity, as indicated by a lack of significant weight loss (Fig. 34B). Therefore, the use of low dose of TA and mithramycin produced significant antitumor activity without toxicity. This suggests that treatment with low dose of mithramycin and TA has a significant therapeutic benefit.

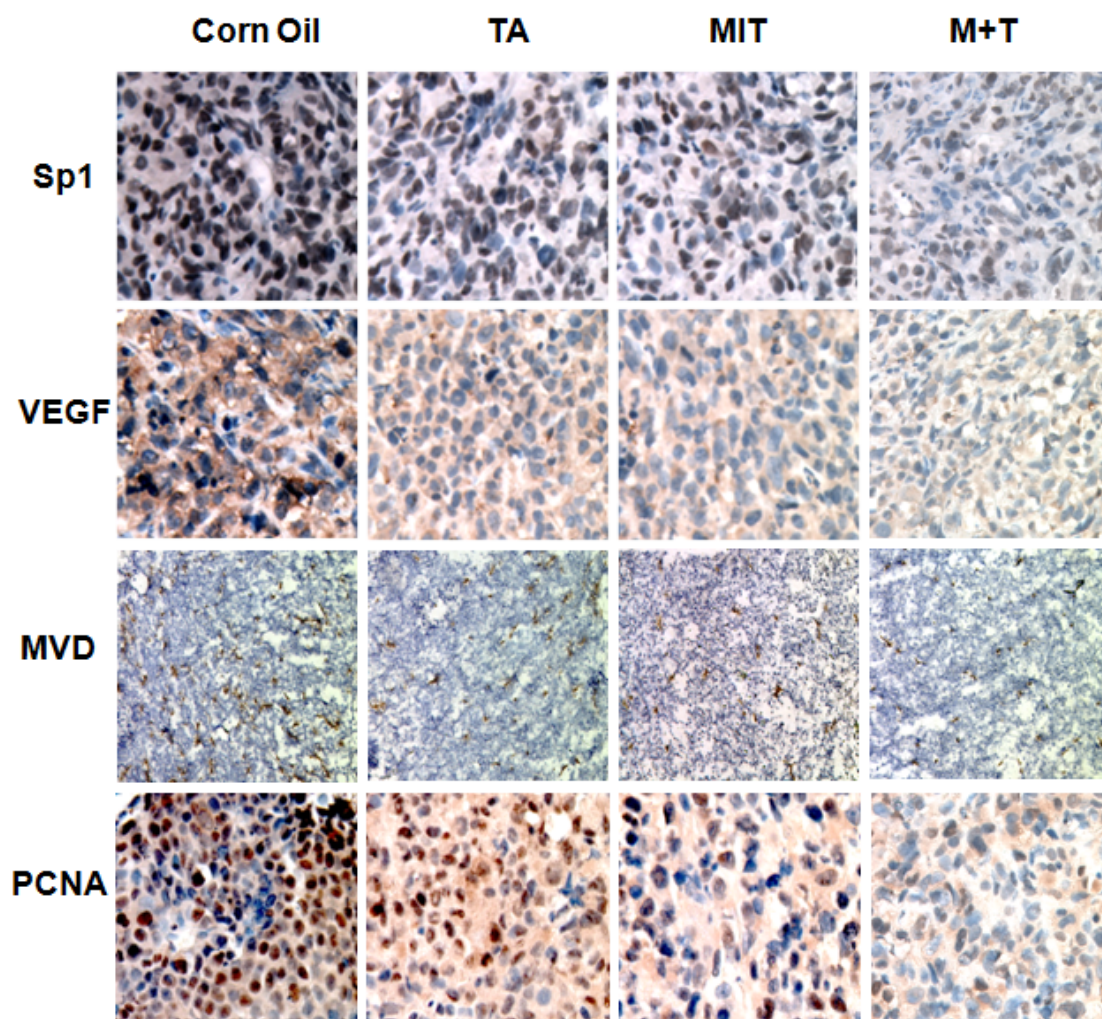


**Figure 34 Synergistic anti-tumor activity of mithramycin and tolfenamic acid *in vivo*** Parallel to Figure 33, a group of mice with PANC-1 tumor were treated with 0.1mg/kg mithramycin and 10mg/kg TA. A1, All the 4 groups of mice tumors were measured once every week, and at each measurement, the mean  $\pm$  SD tumor volume in the five mice in each group was calculated. Tumor weight (A2) and mice body weights (B) were measured at the end of the experiment. A3 and A4, Representative mouse and the tumor from each group. \*  $P < 0.05$  in a comparison between the treated and respective control groups.



### **Effects of mithramycin and tolfenamic acid treatment on gene expression and Sp1 recruitment on Sp1 and VEGF promoters *in Vivo***

To determine the gene expression alternation with treatment of mithramycin and TA, immunohistochemistry staining was performed using the PANC-1 tumor tissue collected from mice that received treatment with PBS, TA, mithramycin, or T+M. As shown in Fig. 35, treatment with TA or mithramycin alone can decrease expression of Sp1 and its downstream molecule VEGF. Tumor microvessel density, shown with CD31 staining, also decreased comparing with control group. Whereas mithramycin and TA combination treatment suppressed Sp1 and VEGF expression dramatically, this was consistent with the much reduced MVD. Besides these, mithramycin and/or TA treatment decreased tumor PCNA protein level, especially in combination treatment. These data suggested that the synergy anti-tumor activity of mithramycin and TA combination may be produced through not only anti-angiogenesis effect, but also the direct growth inhibition of tumor cell proliferation. Indeed, there is also the synergy low-dose combination cytotoxicity, but no cytotoxicity when use alone, *in vivo*.



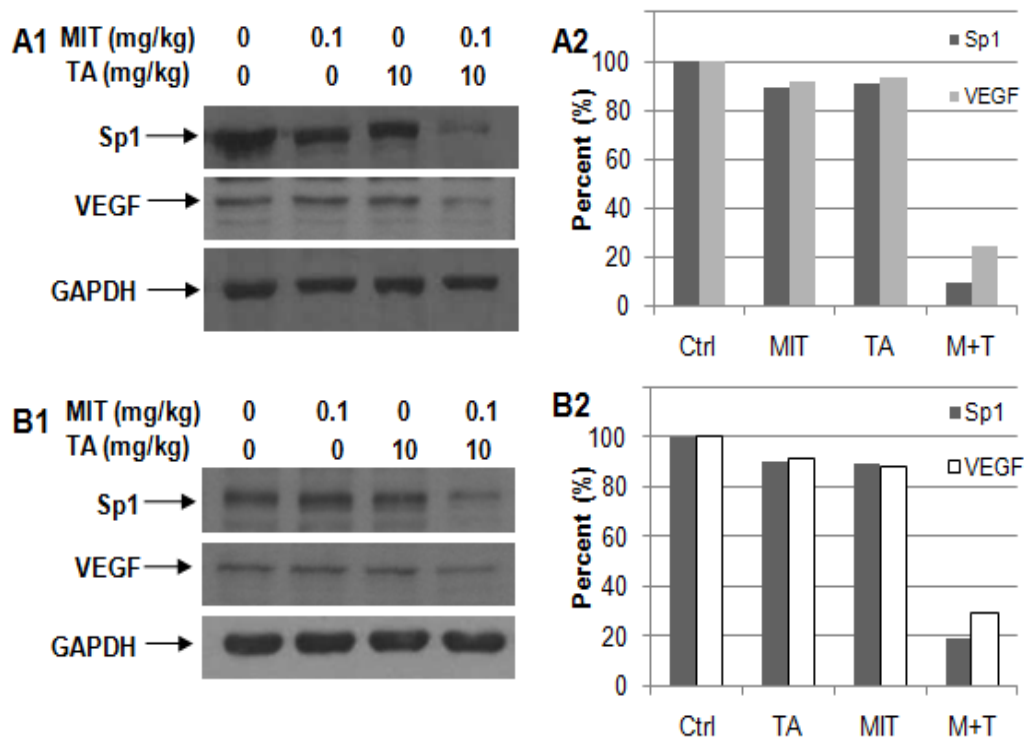
**Figure 35 Analysis of gene expression and microvessel formation in tumor tissues**

The tumor tissues described in Fig. 33 were collected and processed to do immunohistochemistry staining of Sp1, VEGF, PCNA and micro-blood-vessel as described in Materials and Methods.

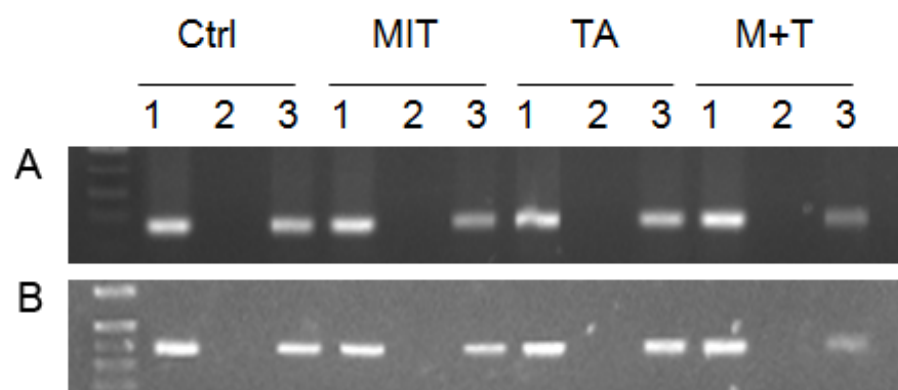
To confirm the Sp1 and VEGF alternation, western blot analysis was then performed. Total protein lysates extracted from the PANC-1 tumor tissue specimens collected from mice that received treatment with PBS, TA, mithramycin, or T+M were used. As shown in Fig. 36A, Sp1 and VEGF protein were down-regulated especially in the combination treatment. BxPC3 tumor tissue collected from mice that received the same treatment was also analyzed (Fig. 36 B).

Then I sought to determine whether Sp1 transcription was regulated by TA and mithramycin treatment *in vivo*. Similar to *in vitro*, mithramycin treatment reduced Sp1 recruitment onto its own and VEGF promoter as shown in the ChIP assay. While treatment with TA has minor affect on Sp1 recruitment to its own and VEGF promoter *in vitro* (Fig. 37). In contrast, treatment with mithramycin and TA suppressed Sp1 protein recruitment to its own promoter significantly.

These data suggested that mithramycin and TA combination treatment synergistic down-regulates Sp1 and VEGF expression and produces tumor suppression effect *in vivo*.



**Figure 36 Effects of treatment with mithramycin and tolfenamic acid on Sp1 protein level** The tumor tissues described in Fig. 34 were collected and processed as described in Materials and Methods. A-B, total protein lysates were harvested from tumor tissues and the level of protein expression in them was determined using Western blot analysis. Equal protein-sample loading was monitored by probing the same membrane filter with an anti-GAPDH antibody. A, PANC-1 xenograft tumors; B, BxPC3 tumors.



**Figure 37 Effects of mithramycin and tolfenamic acid treatment on Sp1 and VEGF promoter *in vivo*** Chromatin Immunoprecipitation was performed as described in Materials and Methods. Chromatin extracted from tumor tissues described in Fig. 34 were collected and processed. A specific anti-Sp1 antibody and oligonucleotides flanking the (A) Sp1, (B) VEGF promoter regions containing Sp1-binding sites was used. Lane 1, input chromatin DNA; lane 2, chromatin DNA with control IgG; lane 3, chromatin DNA with anti-Sp1 antibody.

## DISCUSSION

In this chapter, it was found that metronomic low dose of mithramycin and tolfenamic acid combination synergistic down-regulates Sp1 expression, which is a key positive regulator of various angiogenic factors expression including VEGF and produced synergistic anti-tumor therapeutic effect on xenograft tumor grown in nude mice. This therapeutic effect was consistent with Sp1 activity suppression and the down-regulation of its downstream angiogenic molecules. The experimental results indicate that mithramycin targets Sp1 at its transcription level through competition its recruitment on the Sp1 sites. But tolfenamic acid promotes Sp1 protein degradation. These findings showed researchers the synergistic down regulation of transcription factor Sp1 and would further drive a new rational of drug combination to target a protein molecule at different biological level.

Angiogenesis plays important roles in tumor growth and metastasis. Studies have been shown that both Sp1 and VEGF are important to pancreatic cancer angiogenesis [130, 225]. Currently targeting of VEGF signaling and function is still a hot spot in anti- angiogenesis, growth, and metastasis of most tumor type. Strategies have been developed, including the use of anti-VEGF antibodies, targeting VEGF receptors to directly interfere the signal effect [124, 125]. Additionally, multiple genes, identified in pancreatic cancer switch of the "angiogenic network" [126, 127], were also proposed to be the anti-angiogenic

targets and targeted in experimental mouse model [58, 128]. These reported clinical research results and experimental studies using different approaches showed the crucial role of the angiogenic process in pancreatic cancer. Also, replication deficient recombinant adenovirus mediated was evaluated in human pancreatic cancer mouse model and shown to be efficient gene therapy for pancreatic carcinoma [129].

In methodology, visualized fluorescence imaging was developed in tumor angiogenesis research in nude mice based on the stably green fluorescent protein expression cancer cell lines and mouse models [234, 235]. Recently, multicolored fluorescent proteins were used to develop color-coded fluorescent protein imaging models of tumor angiogenesis [236]. The generally termed AngioMouse can quantitatively determine efficacy of anti-angiogenesis compounds through visualizing the details of the tumor-induced angiogenesis.

As a regulator of the important pro-angiogenic molecules, Sp1 can be regulated by mithramycin through direct competition of Sp1 recruitment on Sp1 sites in Sp1 promoter as it was shown in last chapter. During the regulation, Sp1 protein level change would appear until the mRNA synthesized before mithramycin binds Sp1 sites, which will take certain time. This is consistent with the observation of continuous high dose of mithramycin was needed to obtain significant tumor inhibition which leads to the significant cytotoxicity. Although mithramycin can effectively block Sp1 mRNA synthesis, the abundance and strong stability of Sp1

protein prevents mithramycin from rapid down-regulation of Sp1 protein in the tumor cells. In this chapter, it was demonstrated that tolfenamic acid can promote Sp1 protein degradation. More importantly, combined use of mithramycin and tolfenamic acid, neither of which has significant effects on Sp1 protein expression, led to substantial down-regulation of Sp1 protein, which was consistent with a synergistic antitumor effect in mouse models.

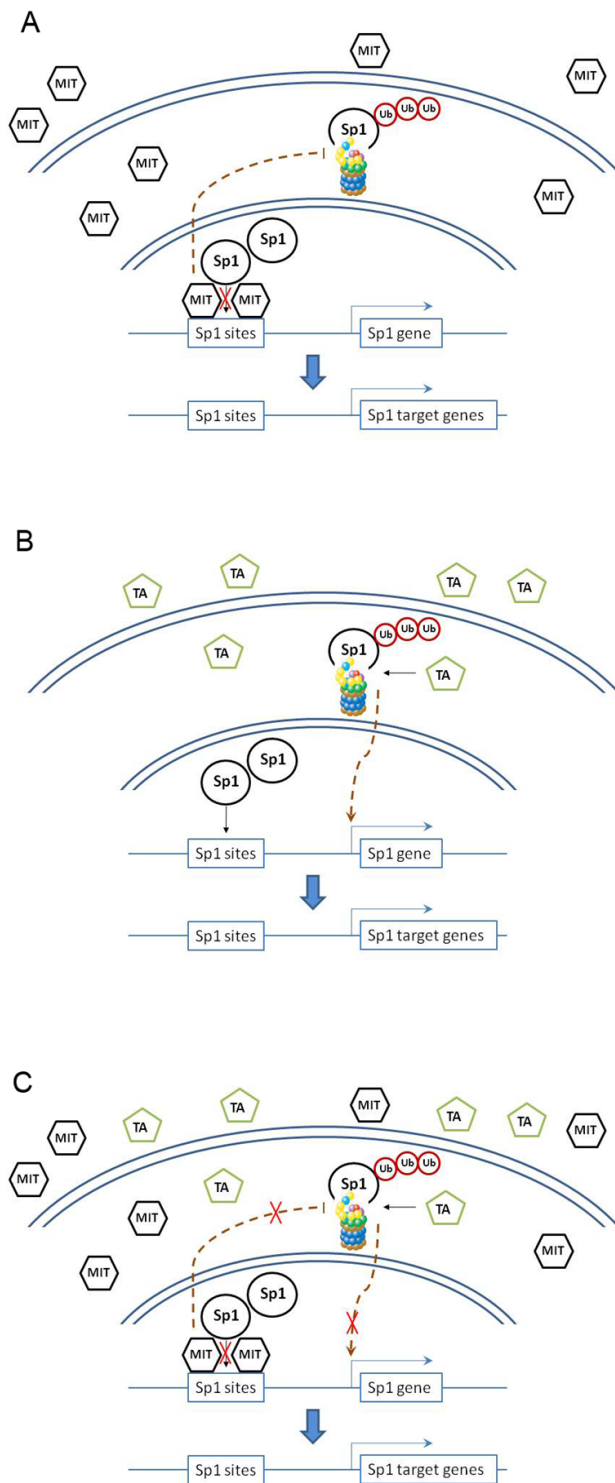
Studies have shown that a number of non-steroidal anti-inflammation drugs have anti-angiogenesis activity in a wide variety of xenograft models. Celecoxib and tolfenamic acid were shown to decrease pancreatic tumor growth and metastasis in nude mice [130, 225]. The drugs activate Sp1, Sp3, and Sp4 protein degradation and inhibit VEGF mRNA synthesis and protein expression in pancreatic cancer cells. Sp1 was reported to be over-expressed in human pancreatic cancer and associated with poor survival [61, 109, 237]. Evidences have been provided that transcription factor Sp1 regulates VEGF expression in pancreatic cancer cells. In Sp protein family, Sp1, Sp3 and Sp4 are more closely related and form a monophyletic group [238]. Experimental results showed that through the activation of proteasome-dependent degradation of Sp protein, tolfenamic acid exhibited growth inhibitory effect by anti-angiogenic strategy. However, down-regulation of Sp1 by mithramycin treatment is mostly involved in transcription repression of Sp1 mRNA. Therefore, mithramycin and tolfenamic acid have distinct mechanisms of actions in regulation of Sp1 expression and



activities, and their interaction forms the molecular basis for their synergistic anti-angiogenesis and antitumor activities.

In addition to its anti-angiogenic function reported, down-regulation of Sp1 also may cause the altered expression of genes important to cell survival, a mechanism that is likely for the antitumor activities of tolfenamic acid and mithramycin. For example, tolfenamic acid treatment activates Sp protein degradation, decreases Sp proteins binding to survivin promoter and inhibits survivin expression in pancreatic cancer cell and subsequently sensitizes the pancreatic cancer cell to radiotherapy [239]. Consistently, the data in this chapter also shows tolfenamic acid inhibits tumor cell growth *in vitro* and the effect was synergized when combined with mithramycin (Table1, 2 and Figure 28, 29). Altered expression of survivin expression may be one of the mechanisms underlying the cytotoxic effect of tolfenamic acid and mithramycin.

Although the data showed tolfenamic acid treatment has minor effect on the recruitment of Sp1 on its own and VEGF promoter (Figure 31). Tolfenamic acid did affect the Sp1 promoter activity and VEGF promoter activity (Figure 30). The possible anti-angiogenic effect of tolfenamic acid may be rely on decreasing total Sp1 protein quality but not the interference of the physical contact of Sp1 and its binding sites in the promoters which needs to be further investigated.



**Figure 38 Schematic model of mithramycin and tolfenamic acid on Sp1**

A, mithramycin treatment physically blocks Sp1 binding to Sp1 sites in its own and its downstream

molecules but may leads to a negative feedback to Sp1 protein degradation

B, tolfenamic acid treatment facilitate Sp1 protein degradation and may cause its trans-activation mRNA transcription

C, mithramycin and TA combination treatment blocks trans-activation and facilitate its degradation which blocks the negative feedbacks and produce synergistic effect

Due to the insoluble character in water, tolfenamic acid was shown to need about 100 times in concentration to have the similar cell growth inhibition affect *in vitro* (Figure 27). The limit leads to the relatively low therapeutic index. Polymer-drug conjugates nanoparticles, one of the major categories of targeted drug delivery system, are synthetic water-soluble polymers used as the drug carriers [240]. Compare with the corresponding parent drugs, the clinical trial results of polymer-drug conjugates nanoparticles have several advantages including decreased side effects, enhanced therapeutic efficacy, ease drug administration, and improved patient compliance. Tolfenamic acid may be more effective by linking with nanocarriers, which brings more drugs to the tumor site and reduces exposure of normal tissues to the drug.

As our experimental results showed mithramycin has significant cytotoxicity when it produces significant antitumor effect as a single drug using mouse body weight change as the measurement. When tolfenamic acid was used together, Sp1 protein was synergistic down-regulated through different level and tumor growth was significant inhibited. But no detectable cytotoxicity was observed. Mithramycin nanoparticle has been developed [241]. Polylactide co-glycoide (PLGA) based mithramycin nanoparticle inhibited RAW264 macrophages and smooth muscle cells and reduced the number of circulating monocytes in rabbits. A nanoparticle with homing moiety actively binding to target cell has been widely demonstrated. It was shown that the interaction makes more drug compounds gathering at the diseased sites, whereas systemic drug exposure was reduced

and subsequently minimized cytotoxicity. One category of the homing moieties is antibody including intact antibody, fragments (Fab' or (Fab)<sub>2</sub>) or genetically engineered Fv. In addition to some certain carbohydrates, transferrin was proposed as a homing moiety. Compare with antibody, its potential advantages is the risk of immune response is rather low because of the present of transferrin in organism in high concentrations [242, 243]. The pharmacodynamics and pharmacokinetics properties of mithramycin encapsulated by nanoparticle with home moiety should be an interesting topic to be investigated.

As our experimental results showed mithramycin has significant cytotoxicity when it produces significant antitumor effect as a single drug using mouse body weight change as the measurement. When mithramycin and tolfenamic acid were used together, Sp1 protein was synergistic down-regulated through different level and tumor growth was significant inhibited. But no detectable cytotoxicity was observed. Our data suggested that the combination of mithramycin and tolfenamic acid could achieve highest therapeutic index.

Collectively, our study suggests that mithramycin competing Sp1 recruitment to Sp1 sites on both Sp1 and VEGF promoters. Tolfenamic acid does not have the competing ability but it down-regulates Sp1 protein level by direct targeting Sp1 at protein level. The metronomic low dose use of mithramycin in combination with tolfenamic acid is a novel strategy of targeting angiogenic molecule Sp1 at both transcriptional and protein degradation level. Combining mithramycin and

tolfenamic acid in cancer clinical studies could represent a rationale step forward.

This double targeting from different level strategy would develop effective targeted therapy for pancreatic cancer and other cancers.

## CONCLUSION

Chapter I of this dissertation demonstrated that knockdown Sp1 protein down-regulates Sp1 downstream angiogenic factors, significantly reduced pancreatic cancer cell angiogenic potential *in vitro* and led to the angiogenic phenotypic change *in vivo*. All of the experimental results suggest that Sp1 is a key factor for pancreatic cancer angiogenesis.

Chapter II shows that Bevacizumab treatment up-regulates Sp1 and VEGF protein in xenograft tumor tissue, which may trigger drug resistance to Bevacizumab in turn. This positive feedback mechanism trans-activate Sp1 and its downstream angiogenic factors' over-expression subsequently. Combination of mithramycin with Bevacizumab produced synergistic antitumor activity by both interposing upstream Sp1 transcriptional regulation and neutralizing downstream effector molecules such as VEGF.

Chapter III shows the experimental evidence that mithramycin competes Sp1 recruitment to Sp1 sites on both Sp1 and VEGF promoters. Tolfenamic acid does not have the competing ability but it down-regulates Sp1 protein level by direct targeting Sp1 protein. The metronomic low dose use of mithramycin in combination with tolfenamic acid is an important novel strategy of targeting angiogenic molecule Sp1 at both transcriptional and protein degradation level.

Collectively, in this dissertation new metronomic anti-angiogenic therapy strategies of pancreatic cancer were designed and tested on mouse models. The novel findings may provide both theoretical and practical direction to clinic practice. The proposed rationales may push the development of effective target therapy step forward for pancreatic cancer and other cancers. Hopefully, the experimental therapeutic designs may be translated to clinic and benefit pancreatic cancer patients in the near future.

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## **VITA**

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