THE THROMBOSIS PATHWAY PROMOTES PANCREATIC CANCER GROWTH AND METASTASIS

by

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A Dissertation

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy

Department of Biological Sciences

West Lafayette, Indiana

May 2019

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Dedicated to my parents, relatives, friends, teachers and a childhood dream

ACKNOWLEDGMENTS

I hold my greatest and deepest gratitude to my mentor, Dr. Stephen Konieczny. He took me into his lab at my most desperate time, saved my dream and raised me towards being a good scientist. I cannot be more grateful to be one of his students. He provided us the best working environment, encouragement, guidance and endless sarcastic teasing. He trained me effortlessly regardless of my ordinary talent, bad habits and artless sense. He created an atmosphere in the lab which allowed me to develop and regain my passion in science and my self-confidence for being a scientist in the future. He taught me lots of skills including writing, experimental design, critical thinking, organizing, etc. However, I feel I still have much more to learn from him. He will be forever remembered as my mentor for his selflessness guidance, as my friend for his endless encouragement, and as my father for his instillation of a scientist soul in me.

I would also like to thank my committee members, Dr. Shihuan Kuang, Dr. Susan Mendrysa, Dr. Elizabeth Taparowsky and my former committee member Dr. Xiaoqi Liu for their support, guidance and critiques of my project. I enjoyed all of the many conversations and discussions we had during my committee meetings which helped me to further advance my professional training. A big thank you to Dr. Matthew Flick who initiated this project and supported me throughout my entire Ph.D. career. He is definitely a good big brother of the SFK lab. He is always passionate and positive about science and we had many fun times working together. A special thank you to Dr. Elizabeth J Taparowsky. In addition to her valuable and insightful suggestions on science, she offered me her honest opinions on multiple areas, including but not limited to politics, education, history, school policy, and her love for mushrooms. Through these conversations with her, I, as a foreigner to this country, embraced multiple aspects of the beauty of the American culture. I also would like to thank Dr. Chang-Deng Hu for his support and

I am touched by his enthusiasm in science. Thank you Dr. Dorothy Teegarden for your guidance and support for improving my communication skills, expanding my knowledge in cancer prevention.

I would like to thank all of the present and past lab members in the lab including Chunjing, Dave, Anju, Rosie, Brad, Patrick and the undergrads Erin, Emily, Sarah M, Sarah B, and Rachel. Anju has the kindest soul and she was always ready to help others. Brad and Patrick are my buddies who were always willing to offer hands, exchange ideas, and provide any necessary help for my project. Also, I will never forget the game nights we had with Brad's hilarious laugh, Amy's enthusiastic participation, Patrick's sarcastic jokes, Connie's warm cookies and Zero's effortless interruptions. All of these will be dearly missed. A big thank you to Dr. Barbara Damsz, the lab mom and my coffee partner. She kept the entire lab well organized and clean. I have learned enormous life skills from her and I will miss her very very much. Another special thank you to my undergrad Erin. She and I shared the same enthusiasm and curiosity for science. I truly enjoyed every minute during our time together and I cannot wait to see how she develops into the person I know she wishes to be.

Thanks to all the friends that I met while at Purdue. Every single one of you formed a unique piece in my Ph.D. career and helped me to complete my life. I cannot list every name here but I remember and miss every one of you.

Last but not the least, I would like to thank my parents: Dajin Yang and Jinghua Lu. They gave me life. They didn't know how to educate me but they were willing to give everything they had to support me for my education and career goals. There was no depth in words or wisdom to share, but the pure unconditional love from them supported me through my toughest times. I only

pray to slow down their aging so that I will have time to pay them back. They made me into the person that I wish to be and I love them all.

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AP-1	Activator protein 1
APC	Activated protein C
ASO	Anti-sense oligonucleotide
CAFs	Cancer associated fibroblasts
CCHMC	Cincinatti Children's Hospital Medical Center
DAPI	4',6-diamidino-2-phenylindole
DOX	Doxycycline
ECM	Extracellular matrix
Erg1	Early growth response protein 1
ERK	Extracellular signal-regulated kinases
F2R	Protease activated receptor 1 gene name
F3	Tissue factor gene name
FBS	Fetal Bovine serum
FVII	Factor VII
GEMM	Genetic engineered mouse models
GM-CSF	Granulocyte macrophage colony stimulating factor
H&E	Hematoxylin and Eosin
HA	Hyaluronic acid
HBSS	Hanks' balanced salt solution
hgDMEM	high glucose Dulbecco's Modified Eagle Medium
HIF-1 α	Hypoxia-induced factor 1α
HMG	High mobility group
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MDSCs	Myeloid derived suppressor cells
NFκB	Nuclear factor kappa B
PanIN	Pancreatic intraepithelial neoplasia
PAR-1	Protease activated receptor 1
PARs	Protease activated receptors

PDAC	Pancreatic ductal adenocarcinoma
PDX1	Pancreatic and duodenal homeobox 1
PI3K	Phosphoinostitide 3-kinase
PKC	Protein Kinase C
PNET	Pancreatic neuroendocrine tumors
PSC	Pancreatic stellate cells
SHH	Sonic hedgehog
SPARC	Secreted protein acidic and rich in cysteine
TAFI	Thrombin-activated fibrinolysis inhibitor
TAM	Tumor associated macrophages
TBST	Tris-buffered saline
TF	Tissue factor
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor alpha
TWIST1	Twist family BHLH transcription factor 1
VEGF	Vascular endothelial growth factor
VTE	Deep venous thrombosis

ABSTRACT

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Degree Received: May 2019
Title: The Thrombosis Pathway Promotes Pancreatic Adenocarcinoma Tumor Growth and Metastasis
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Pancreatic ductal adenocarcinoma (PDAC) is an incredibly lethal disease with a 5-year survival rate of less than 8 percent in the United States due to a lack of viable treatment options. The failures of chemo- and radiotherapies have been linked to the heterogeneous nature of the tumor microenvironment which forms a hypovascular, immunosuppressive and high coagulation activity tissue. Indeed, PDAC patients have one of the highest rates of thrombosis complications among all cancer types. The expression of two key coagulation factors, Tissue Factor (TF) and Protease Activated Receptor 1 (PAR-1), have been associated with poor patient prognosis and aggressive cancer progression. However, the molecular roles/mechanisms of TF and PAR-1 in PDAC progression are not known. To establish how clotting factors (PAR-1, TF) influence PDAC tumor progression, I utilized a genetically modified mouse model (KPC) where $KRas^{G12D}$ and $TRP53^{R172H}$ mutations were specifically introduced into mouse pancreas acinar cells to initiate PDAC progression. Multiple primary mouse PDAC cell lines were generated and characterized. TF and PAR-1 were highly expressed in primary KPC pancreatic lesions, in PDAC tumors, and in KPC-derived cell lines, an expression profile that is also observed in PDAC patient biopsies. In allograft studies, tumor growth and metastatic potential were significantly diminished by shRNA reduction of TF or PAR-1 in cancer cells or by genetic or pharmacological reduction of the coagulation zymogen prothrombin in mice. Notably, PAR-1 deleted KPC cells (KPC-Par-1^{KO}) failed to generate sizable tumors; a phenotype completely rescued by restoration of PAR-1

expression. To test the significance of targeting PAR-1 in a clinical setting, PAR-1 expression was withdrawn from established tumors to mimic a potential inhibitory effect of PAR-1 on solid PDAC tumors. Removal of PAR-1 from tumors (11 days post injection) yielded a diverse effect on tumor growth which can be categorized into (i) a decline in tumor growth; (ii) continued tumor growth; and (iii) stagnant tumor growth. Immunohistochemistry analysis of KPC2 shCon vs. shPar-1 subcutaneous allograft tumor samples revealed a massive immune cell infiltration in KPC2 shPAR-1 tumors when compared to KPC2 shCon control tumors. Accordingly, KPC-Par-1^{KO} cells failed to form tumors in immune-competent mice but displayed robust tumor growth in immune-compromised *NSG* mice, providing the first evidence of a PAR-1 mediated tumor immune evasion pathway operating in PDAC.

Together, these results demonstrate that PDAC disease is driven by activation of the coagulation system through tumor cell-derived TF, circulating prothrombin, and tumor cell-derived PAR-1. These studies also highlight a novel mechanism by which thrombin/PAR-1-mediated tumor growth involves suppression of anti-tumor immunity in the tumor microenvironment.

CHAPTER 1. INTRODUCTION

1.1 The Pancreas

1.1.1 *Pancreas development and cell types.*

The pancreas is a complex organ which serves a dual role in metabolism – regulating blood glucose levels and the production of digestive enzymes for the small intestine. Initial development of the organ occurs early in embryogenesis (E9.5 for mice; E26 for human) where two distinct lobes (dorsal and ventral buds) emerge from the gut endoderm. In both species the two pancreatic buds elongate along the presumptive duodenum (Kallman & Grobstein, 1964; Pictet et al., 1972), and upon gut rotation during development, the lobes fuse to form a single, intact pancreas. Shortly after, the pancreas epithelium cells expand and differentiate into endocrine islet cells and exocrine acinar and duct cells with a specific topological organization where the acinar cells form acini clusters at the end of ductal networks and endocrine cells aggregate throughout the organ near blood vessels (**Figure 1.1**). During these processes, key signaling pathways and transcriptional networks guide the specificity of pancreatic cell differentiation. First, the prevalent expression of sonic hedgehog (*SHH*) near the gut endoderm is selectively excluded from the pancreas area. Genetically engineered mouse (GEM) lines have revealed that the exclusion of SHH signaling allows expression of the pancreas-specific transcription factor pancreatic and duodenal homeobox 1 (PDX1) which induces the epithelial cells become to pancreatic progenitor cells (Apelqvist et al., 1997). After the fusion of the dorsal and ventral buds, several other key transcription factors expressed in the progenitor cell pool help to specify different pancreas cell types. Specifically, the bHLH family member PTF1a and the high mobility group box (HMG) - domain containing factor SOX9 control the differentiation of acinar cells and duct cells, respectively. GEM experiments have demonstrated that mice lacking any of these key transcription factors during development

display degrees of hypoplasia or pancreatic agenesis (Reviewed in Gittes 2009; Pan and Wright 2011). Although PTF1a is essential for acinar cell development, PTF1a alone is not sufficient to effectively induce complete acinar cell differentiation. MIST1, another bHLH transcription factor, is highly expressed in the embryonic and adult acinar cells and loss of *Mist1* leads to defects in cellular polarity and regulated exocytosis where the pancreas produces greatly reduced levels of secreted digestive enzymes (Direnzo et al., 2012; Pin et al., 2001). Therefore, both transcription factors, PTF1a and MIST1, are critical for maintaining acinar cell identity.

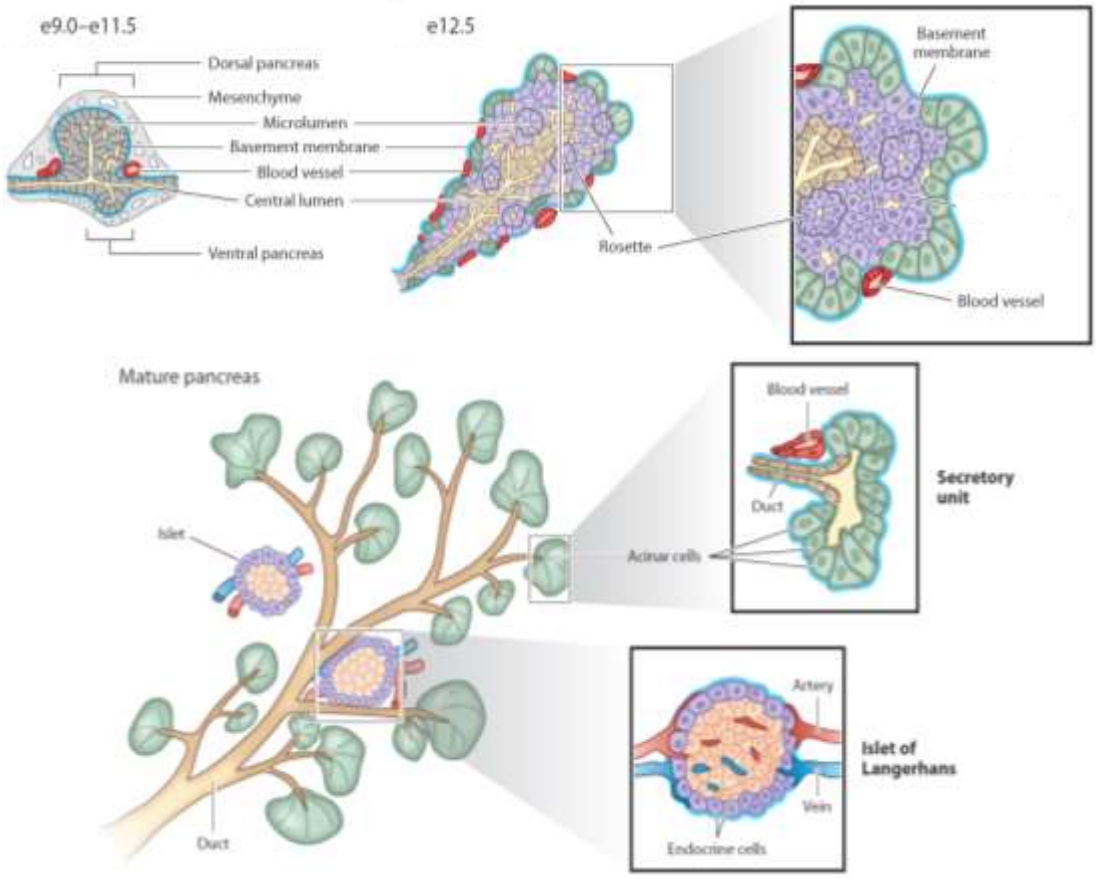

Figure 1.1 Pancreas development. The dorsal and ventral parts of the developing pancreas fuse together at E12.5 to form the intact pancreas. The pancreas epithelium cells continue to expand and differentiate into endocrine, acinar and ductal cells with a specific topological organization where the acinar cells form acini clusters at the end of ductal networks and endocrine cells aggregate into islets throughout the organ near blood vessels. The figure is adapted from (Hung Ping Shih, 2013).

The endocrine pancreas consists of the Islets of Langerhans, which comprise approximately 5% of the organ mass. Despite their modest numbers, islets are critical for blood glucose metabolism as the cells secrete key hormones into the circulation system. The islets consist of five different cell types where each cell type produces and secretes a single specific hormone: alpha cells – glucagon; beta cells – insulin; delta cells – somatostatin; epsilon cells – ghrelin; gamma cells – pancreatic polypeptide (Da Silva Xavier, 2018). The major hormones that regulate glucose metabolism are glucagon and insulin. Glucagon promotes the breakdown of glycogen in the liver and increases the blood glucose level. Insulin, on the other hand, promotes the uptake of blood glucose into the liver to decrease the blood glucose level. Dysregulation of these two hormones will result in pathological disorders such as hyperglucagonemia (glucagon) or diabetes (insulin) (DeFronzo et al., 2015).

The remaining portion (95%) of the pancreas makes up the exocrine compartment which consists of acinar cells, centroacinar cells and duct cells. Pancreatic acinar cells are the functional unit of the exocrine compartment, producing and secreting large amounts of inactive hydrolytic zymogens such as amylase, carboxypeptidase, elastase and trypsin into the ductal network, which transports the zymogens into the duodenum. These proenzymes are activated in the duodenum by a proteolytic cleavage event. Upon activation the pancreas enzymes assist in food digestion. Dysregulation of the synthesis or secretion of these zymogens leads to health disorders including pancreatitis or pancreatic insufficiency. Although mild episodes of pancreatitis can be treated, the condition itself is a risk factor for pancreatic cancer. Patients who develop acute pancreatitis, chronic pancreatitis or hereditary pancreatitis have a 66-, 16- and 69-fold increased risk of developing PDAC, respectively (Lowenfels et al., 1993; Munigala et al., 2014; Raimondi et al., 2010). Interestingly, upon pancreatitis inflammation, the key transcription factors PTF1a and

MIST1 are silenced. The silencing of these regulators leads to acinar cells to dedifferentiate back to a duct-like cell fate in which digestive enzyme production is shut down to allow the tissue damage to be repaired. Once the tissue damage is cleared, the duct-like cells differentiate back to the acinar lineage. This process is called acinar-to-ductal metaplasia (ADM) (Reichert & Rustgi, 2011). To sum up, pancreas development is tightly controlled by multiple signaling and tissue-specific transcription factors and disruption of key pathways can result in significant abnormalities in the adult organ.

1.1.2 *Pancreatic cancer cell origin and genetics.*

Pancreatic cancer can be largely categorized into two groups: endocrine tumors and exocrine tumors. Endocrine tumors (also known as pancreatic neuroendocrine tumors (PNETs)) are very rare, accounting for less than 5% of the pancreatic cancer cases in the U.S. with an estimated frequency of 1-4 cases per 1,000,000 individuals (Halfdanarson et al., 2008). In contrast, exocrine pancreatic cancer is the most prevalent form and is a clinically challenging disease. The vast majority of exocrine pancreatic cancer (95%) cases are categorized as pancreatic ductal adenocarcinoma (PDAC), where the current 5-year survival rate is a dismal 8% (Siegel et al., 2018). An estimated 55,000 new cases are diagnosed each year with an associated 44,000 deaths.

There are many reasons for the poor prognosis for PDAC patients, including late stage diagnosis, the early metastatic properties of the disease, and the ability of PDAC to resist conventional chemo- and radiotherapies. At the molecular level, pancreatic cancers harbor oncogenic mutations with unusual high frequency, with over 90% patients exhibiting *KRas* mutations (Bamford et al., 2004; Kim et al., 2011), 70% patients with *p53* mutations (Hwang et al., 1998) and over 50% patients with *p16* mutations (Chen et al., 2009; Schutte et al., 1997). Multiple GEM models expressing specific oncogenes in different cell types have been developed

to aid in uncovering the basic molecular biology of PDAC. Importantly, these mouse models have proven to be faithful in reproducing the biochemical, immunological and histological features of human PDAC (Habbe et al., 2008; Hingorani et al., 2003; Shi et al., 2009). From these studies, investigators have identified that acinar cells and duct cells of the exocrine compartment can each serve as the "cell of origin" for PDAC (Bailey et al., 2016; Habbe et al., 2008; Hingorani et al., 2005; Principe et al., 2018; Shi et al., 2009). In most GEM models, KRas mutations (G12D or G12V) have been used and shown to be the primary molecular PDAC initiator and required for tumor progression (Collins et al., 2012; di Magliano & Logsdon, 2013). Indeed, *KRas* mutation alone is sufficient to initiate PDAC progression under associated tissue damage or inflammatory stress (e.g., Pancreatitis). Importantly, mutant KRas is required for maintaining the pancreatic cancer phenotype since removal of KRas mutations often results in tumor regression (Collins et al., 2012). *Trp53* (R172H) mutation and *Cdkn2a* (p16) deletion have been used in combination with KRas mutations in acinar cells and have been shown to accelerate PDAC progression, revealing how multiple genetic events can dramatically influence tumor formation and progression (Aguirre et al., 2003; Bailey et al., 2016). In all cases, PDAC oncogenic transformation events provoke the deregulation of multiple downstream signaling pathways including Hedgehog signaling (Pasca et al., 2006), Notch signaling (Miyamoto et al., 2003) and EGFR signaling (Ardito et al., 2012), observations that are consistent with the human disease (Mihaljevic et al., 2010; Morris et al., 2010).

Combined with pathology observations, investigators have proposed a pancreatic cancer progression model where under normal conditions adult acinar cells transiently de-differentiate into ductal-like cells upon short-term stress, such as local inflammation. After a short recovery period, the duct-like acinar cells differentiate back to normal acinar cells with full restored function.

However, upon mutated KRas activation and sustained KRas signaling, acinar cells undergo a "permanent" ADM switch which eventually develops into pancreatic intraepithelial neoplasia (PanIN). PanINs subsequently obtain additional key mutations (e.g., TP53, Cdkn2a) on their way to progressing to PDAC (**Figure 1.2**).

1.1.3 *Pancreatic cancer treatment*

Due to the lack of early detection, the majority of pancreatic cancer patients are diagnosed at a very late stage where the tumor is unresectable, greatly limiting treatment options. Indeed, less than 20% of PDAC patients are diagnosed sufficiently early in the disease to be candidates for surgery. However, even when surgery is an option, most patients (>90%) relapse and die of their disease with limited benefits of survival. To improve survival outcomes, adjuvant therapies have been developed where the standard of care for resectable tumor patients is now surgery followed by adjuvant gemcitabine (a cytidine analog) plus capercitabine (a thymidine synthase inhibitor) treatment. For these patients, the median survival is 26 months with 30% of patients exhibiting a 5-year survival rate (Neoptolemos et al., 2017). For the remaining patients (>80%) with unresectable tumors, Gemcitabine has been the frontline FDA approved chemotherapy drug for the majority of PDAC patients, providing only marginal 1.7 months survival benefits (Burris et al., 1997). Another combination chemotherapy drug, FOLFIRINOX (Combination of 5-FU, leucovorin, irinotecan and oxaliplatin), has more recently come to the forefront and is now recommended by the American Society of Clinical Oncology Clinical Practice Guideline for treating pancreatic cancer patients with metastasis. However, the ideal patients have to be in good physical condition due to significant and severe side effects (Sohal et al., 2016). Despite progress, patients on FOLFIRINOX still exhibit a dismal prognosis with only an average of 11 months median survival (Conroy et al., 2011).

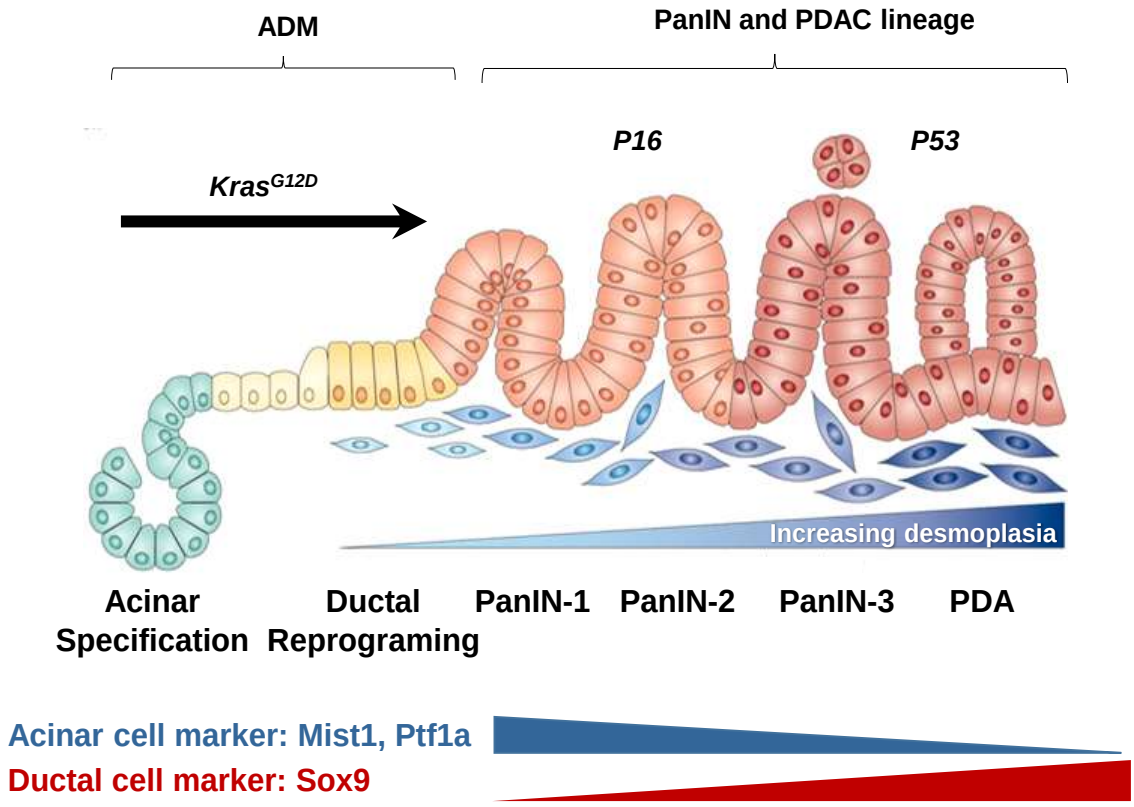

Figure 1.2 Pancreatic cancer progression diagram. Upon *KRas* mutation, acinar cells undergo irreversible dedifferentiation and eventually develop into pancreatic intraepithelial neoplasia (PanIN). PanINs subsequently obtain additional key mutations (e.g., *TP53*, *Cdkn2a*) on their way to progressing to PDAC. The figure is adapted from (Ralph H. Hruban, 2000).

Numerous other targeted therapies have also been evaluated on PDAC patients with or without combination of gemcitabine therapy, including vascular endothelial growth factor inhibitors (Hedy Lee Kindler et al., 2010), multikinase inhibitors (Hedy L Kindler et al., 2011; Hedy Lee Kindler et al., 2012), anti-insulin-like growth factor 1 receptor antibody (Fuchs et al., 2015), phosphoinostitide 3-kinase (PI3K) inhibitors (O'Neil et al., 2015) and EGFR inhibitors (Moore et al., 2007). Unfortunately, the only targeted drug that has shown significant, yet marginal, improved survival benefit is the combination of EGFR inhibitor plus gemcitabine with two weeks increased survival over gemcitabine alone.

The failures of these targeted therapies are at least in part due to the hypovascular nature of the surrounding stroma area that is caused by a robust desmoplastic and extracellular matrix. Up to 80% of the PDAC tumor mass can be made up of this dense matrix, greatly limiting the delivery and effectiveness of drugs (G. C. Chu et al., 2007; Michl & Gress, 2013; Neesse et al., 2018). Therefore, alternative combinational therapies that target stroma have been developed in recent years including (i) SHH inhibitors that target the stromal desmoplasia by inhibiting stromal cancer-associated fibroblasts (Olive et al., 2009; Rhim et al., 2014) and (ii) hyaluronidase (PEGPH20) that digests the major extracellular matrix component hyaluronic acid and enhances drug delivery (Hingorani et al., 2015; Jacobetz et al., 2013; Provenzano et al., 2012). The SHH inhibitors initially showed encouraging results in mice as acute treatment with the SHH inhibitor IPI-926 resulted in enhanced gemcitabine delivery and extended survival (Olive et al., 2009). However, in an actual clinical trial using a SHH inhibitor to remove stromal cancer-associated fibroblasts the outcome was not promising. In these patients treatment with the SHH inhibitors led to increase the aggressiveness of the PDAC tumors. Follow up mouse studies showed that cancer associated fibroblasts act to restrain PDAC tumor growth and thus eliminating these cells by prolonged inhibition of SHH signaling promotes PDAC progression (Rhim et al., 2014). The other study testing hyaluronidase PEGPH20 has shown increased efficacy when treated patients were treated with both gemcitabine and PEGPH20, In this case, patients showed a 3 months increased survival rate over gemcitabine treatment alone (Hingorani et al., 2015, 2017). However, this study cannot be reproduced by combining PEGPH20 and the other effective chemotherapy drug FOLFIRINOX where there is no overall survival benefits of PEGPH20 plus FOLFIRINOX treatment (Ramesh K. Ramanathan, 2018), raising concerns on the basic principle of employing a hyaluronic acid targeting strategy. Overall, the stromal targeting strategies in clinical trials and

mouse models have shown some encouraging results that targeting tumor stroma provides better drug delivery, enhanced effectiveness of chemotherapy drugs, and therefore prolonged survival. However, the knowledge gap on the PDAC stromal components halts us from identifying and designing better therapeutic strategies.

After years of failures in clinical trials, the importance of understanding the stromal components in the PDAC tumor microenvironment (TME) has been highlighted for our basic science research and key questions remain, including: (i) what are the roles of each stroma component in enhancing PDAC disease, tumor growth, and metastasis?, (ii) what are the molecular mechanisms by which dysplasia stromal and PDAC cells contribute to PDAC development?, and (iii) what are the molecular mechanisms by which oncogenes and tumor suppressor genes (KRas, TP53) influence specific downstream signaling pathways to effect stromal dysplasia?

1.2 Coagulation Factors and Cancer

1.2.1 *Thrombosis complication and pancreatic cancer*

Thrombotic complications are frequently associated with cancer and are a leading cause of death for cancer patients, second only to infection (Ambrus et al., 1975; Trousseau, 1865). The common thrombotic diseases in pancreatic cancer patients include deep venous thrombosis (VTE), pulmonary embolism, disseminated intravascular coagulation, portal vein thrombosis and arterial thromboembolism (Khorana & Fine, 2004). The robust activated thrombotic diseases in pancreatic cancer patients are thought to ultimately promote disease progression. Using VTE as an example, PDAC has the highest rate of cancer-associated VTE when compared to other cancer types (Horsted et al., 2012; Lyman, 2011). Indeed, VTE is highly correlated with disease aggressiveness (Lee et al., 2013; Mandalà et al., 2007; Menapace et al., 2011; Sorensen et al., 2000). VTE in PDAC patients is associated with an abnormal activated coagulation cascade and robust release of

coagulation factors by tumor cells. The role of the coagulation factors was first thought to regulate tumor angiogenesis (Carmeliet, 2001), but later studies have revealed their roles in promoting tumor cell proliferation, tumor cell migration, invasion and chemoresistance (Palumbo et al., 2007; Queiroz et al., 2014; Tieken et al., 2016).

In the classical view of the coagulation cascade, Tissue Factor (TF) initiates blood coagulation by binding to Factor VII (FVII) and subsequently activating Factor X (FX) when exposed to blood (**Figure 1.3**). These three factors together form a protease complex in the center of the coagulation cascade. The protease complex then cleaves prothrombin which results in producing the activated major coagulation protease, thrombin. Thrombin cleaves fibrinogen into fibrin monomers, which crosslink and form the functional insoluble blood clot. Despite this central role for thrombin, it also has the ability to induce intracellular signaling cascades through specific cell receptors called protease activated receptors (PARs) found on the cell surface of a number of different cell types. For instance, thrombin activates PAR-1 on the cell surface of platelets to induce its procoagulant activity (Andersen et al., 1999) (**Figure 1.3**). Nonetheless, the coagulation cascade and individual coagulation factors exert influence over different cell types even outside the standard role of clot formation.

1.2.2 *Tissue Factor*

Tissue factor (TF), also known as Factor II (F2 as its gene name), is the initiator of the coagulation cascade, providing vital protection to organs prone to chemical or mechanical injury. TF is a 46 kDa, 263 amino acid membrane bound glycoprotein that consists of extracellular, transmembrane and cytoplasmic domains (Petersen et al., 1995). TF is mainly expressed by the sub-endothelial cells surrounding the blood vessel that serve as a hemostatic envelope for potential bleeding. High expression of TF is found in vascularized organs, such as the brain, lungs and heart.

Low expression of TF is found in smooth muscle cells that comprise the vessel wall and in macrophages, where TF activity is greatly enhanced upon activation of the cells. In addition to the outer space surrounding blood vessels, circulating TF, also known as blood-borne TF, is found in monocytes, macrophages and granulocytes (Osterud & Bjorklid, 2006). Generally speaking, TF expression becomes upregulated in response to vascular injury or environmental stress, such as inflammation (e.g., Lipopolysaccharides), tumor necrosis factor alpha (TNF-α), and in hypoxia conditions (A. J. Chu, 2005). Molecularly, TF expression is induced by activation of intracellular signaling kinases including protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinases (ERK) as well as by a select set of transcription factors including activator protein 1 (AP-1), nuclear factor kappa B (NFκB) and early growth response protein 1 (Erg1) (Reviewed in A. J. Chu 2005).

TF is a member of the Class II cytokine receptor superfamily and its extracellular domain shares homologous structure with interferon receptors. The extracellular domain contains a Factor VII (FVII) binding site and upon binding to TF, FVII becomes a biologically active protease that catalyzes the downstream coagulation pathway. The intracellular domain of TF can undergo serine phosphorylation at Ser253 and Ser258 which also affects TF downstream signaling (C. Li et al., 2008).

TF is overexpressed in many cancers, including ovarian cancer (Uno et al., 2007), breast cancer (Ueno et al., 2000), non-small cell lung carcinoma (Regina et al., 2008), prostate cancer (Kaushal et al., 2008), melanoma (Kirszberg et al., 2009), gastric cancer (Wojtukiewicz et al., 2003), leukemia (Hair et al., 1996) and pancreatic cancer (Khorana et al., 2007). Cancer cell-derived TF is mainly thought to initiate the coagulation cascade leading to thrombosis complications that are typically found in cancer patients. However, TF itself can mediate cancer

cell signaling events through TF-Factor VIIa complex formation and subsequent activation of protease activated receptor 2 (PAR-2) (X. et al., 2004).

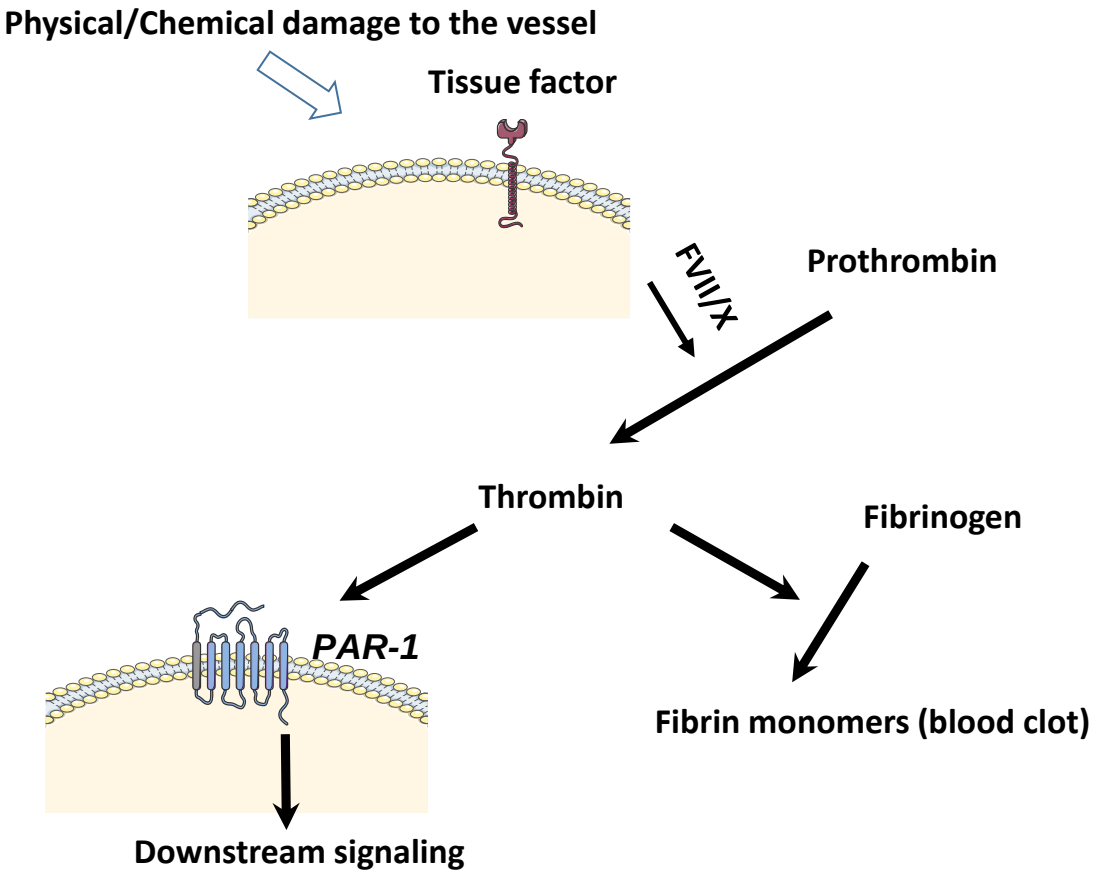

Figure 1.3 Simplified diagram of the coagulation cascade. Upon physical or chemical damage of blood vessels, TF initiates blood coagulation by binding to FVII and subsequently activating FX. The protease complex (TF/FVII/FX) then cleaves prothrombin, resulting in activation of the major coagulation protease, thrombin. Thrombin cleaves fibrinogen into fibrin monomers, which crosslink and form the functional insoluble blood clot. Thrombin also activates the cell surface receptor PAR-1 to trigger downstream signaling.

The PAR-2 receptor complex has been linked to numerous signaling pathways that drive tumor progression, including ERK1/2, JNK, PI3K, and PKCα (Bluff et al., 2008; Hu et al., 2013; Khorana et al., 2007; Rak et al., 2006; Schaffner & Ruf, 2009; H H Versteeg et al., 2000). TF can also exert cell effects by engaging β1 integrins where modulation of integrin binding function and

integrin-driven signaling is controlled in part by phosphorylation of the short TF-cytoplasmic tail (Belting et al., 2004). Given the multiple roles of TF in cancer progression, inhibition or targeting of TF and related pathways have been explored as possible therapeutics in mouse and human cancer models. Indeed, inhibition of TF results in decreased tumor growth and metastasis (Hembrough et al., 2003; Ngo et al., 2007; Henri H Versteeg et al., 2008). However, there are potential concerns of targeting TF such as an increase in internal bleeding, which has been observed in cancer patients treated with Warfarin, a strong anticoagulant (Zacharski et al., 1984). Nonetheless, strategies exist to develop treatment for specific TF functions without affecting the pro-coagulant activity. For instance, Versteeg et al. (2008) developed a specific antibody targeting the TF-FVIIa and PAR2 interactions in the absence of inhibiting the pro-coagulant activity. Interestingly, this antibody successfully suppressed breast tumor growth in a human xenograft model (Henri H Versteeg et al., 2008), suggesting such approaches may be worth pursuing.

Overall, TF exerts multiple functions in promoting cancer progression, including the classical coagulation cascade. However, the extent and pathway(s) by which TF promotes cancer progression in different cancers might be different. Understanding the role of TF in each cancer type is important in designing more effective and safe therapeutics to treat patients with a wide subset of neoplasia.

1.2.3 *Thrombin*

Prothrombin is a glycoprotein zymogen synthesized by liver cells, secreted and found circulating in the blood stream. Cleavage of prothrombin leads to the proteolytically active form thrombin. The process is initiated by FXa which cleaves prothrombin at R320 to generate the intermediate, meizothrombin, and then at R271 to generate the mature enzyme thrombin (Krishnaswamy, 2013). Thrombin is a unique protease generated in the coagulation cascade. Once

proteolytically activated it consists solely of a serine protease domain which allows it to freely diffuse and encounter dozens of protein substrates. Crystal structure studies have revealed that thrombin is highly homologous to other serine proteases including chameleon (Bode, 2005; Bode et al., 1992). Given that thrombin only contains a protease domain, additional features of this protease are required for its specificity. Thrombin contains two anion binding exosites which are made up of groups of surface basic residues. These charged regions interact specifically with the negatively charged region of target substrates (Reviewed in Huntington 2005). Another important feature is that thrombin contains a Na+ binding site (Page et al., 2005; Pineda et al., 2004). Binding of Na+ drastically changes the activity of thrombin where Na+ bound thrombin undergoes a conformational change that increases the accessibility of small substrates to its catalytic site, and in particular favoring procoagulant substrates including fibrinogen, Factor VIII and PAR-1 over the anticoagulant protein C. Blood Na+ concentration is about 140 mM which is slightly higher than the affinity of the Na+ binding site for this ion. Thus, under normal conditions, the majority of circulating thrombin molecules bind Na+, favoring the procoagulant activities.

Thrombin exerts multiple functions by activating numerous substrates on both sides of the coagulation cascade: procoagulation and anticoagulation. On the procoagulation side, thrombin activates, FV, VIII, FXIII, FXI and PARs and also cleaves fibrinogen alpha-chains to produce fibrin, initiating fibrin polymerization. On the anticoagulation side, thrombin activates Protein C, thrombin-activated fibrinolysis inhibitor (TAFI) and antithrombin. As a counter balance of the procoagulant activity, thrombin that escapes the hemostatic plug can bind to thrombomodulin on the endothelial cell surface. The thrombin-thrombomodulin complex directs thrombin to cleave Protein C at R169 and subsequently release the activated form of Protein C. Activated Protein C (APC) inhibits FVa and FVIII by proteolytic cleavage (Esmon, 1993). Another anticoagulant

circulating substrate of thrombin is the carboxypeptidase TAFI. Thrombin cleaves TAFI R92 and releases the activated form of TAFIa. TAFIa then inhibits fibrinolysis and stabilizes the fibrin clot by cleaving the terminal lysine residues from fibrin (Bouma et al., 2001). Interestingly, TAFI shares sequence homology and function with the carboxypeptidases produced by pancreatic acinar cells, especially carboxypeptidase B (Bouma et al., 2001). Given that acinar cells often undergo apoptosis and release their zymogens under inflammation conditions, such as pancreatic cancer. Carboxypeptidase B may contribute to the extreme fibrotic TME of this lethal disease. To maintain normal blood flow, antithrombin circulates in the blood at high concentrations. Once thrombin cleaves R393 of the reactive loop of antithrombin, thrombin forms an irreversible complex with antithrombin which allows it to be cleared from the circulation (W. Li et al., 2004).

Under pathological conditions, many of the effects of thrombin are involved in newly formed blood vessels which requires procoagulant activity. Thrombin simulates the release of vascular endothelial growth factor (VEGF) from platelets (Maloney et al., 1998; Mohle et al., 1997). In cancer, thrombin dramatically increases the metastatic potential of tumor cells through multiple mechanisms including by upregulating integrin expression on the cell surface to enhance cancer cell adhesion to the extracellular matrix (Kanemaru et al., 2012; Wojtukicwicz et al., 1992), triggering the release of PDGF that promotes cell proliferation and migration (Daniel et al., 1986), and by inducing expression of the Twist family of BHLH transcription factors via hypoxia-induced factor 1α (HIF-1α), which contributes to epithelial to mesenchymal transitions and promotes metastasis (Chang et al., 2011; Wallerand et al., 2010).

1.2.4 *Protease activated receptor family*

There are a total of four family members in the protease activated receptor family (PAR1-4), which is part of the G protein coupled receptor superfamily. Unlike classic G protein coupled

receptors, PARs are activated through a proteolytic event at their N terminus that exposes a tethered ligand and allows it to bind back to the transmembrane domain to effect ligand reception signaling. This proteolysis event is irreversible and the PARs undergo endocytosis and lysosomal degradation following activation (Booden et al., 2004; Trejo et al., 1998). Each PAR family member overlaps a portion of their downstream signaling events, although each member also has a unique role in response to specific pathological conditions.

PAR-1

Thrombin mainly exerts its function through activating PARs (PAR-1, 3 and 4) on the cell surface. Most studies to date have focused on PAR-1 mediated signaling. In 1991, PAR-1 was first discovered and cloned by screening a cDNA library generated from cells treated with thrombin (Vu et al., 1991). Thrombin binds to $LDPR^{41}S^{42}$ sequences of the N-terminus of PAR-1 and cleaves between R41 and S42, resulting in PAR-1 receptor activation and coupling with downstream Gαq, Gαi, $G\alpha_{12/13}$ and Gβγ proteins (**Figure 1.4**). Different G proteins guide different downstream signaling. Coupling with Gαq leads to activation of the MAPK pathway and increased Ca2+ flux. Coupling with Gαi leads to inhibition of adenylyl cyclase, while coupling with $G\alpha_{12/13}$ activates the small G proteins, Rho and Rac. Lastly, Heterotrimers composed of PAR-1 and Gβγ promotes the PI3K pathway (Coughlin, 2005; Martin et al., 2001) (**Figure 1.4**).

Although thrombin is the major protease responsible for the activation of PAR-1, other proteases or protease contained complexes have been discovered that are capable of cleaving the N-terminus of PAR-1 to elicit cellular functions. These include trypsin, activated protein C (APC), FVII-TF complex, granzyme A and plasmin. However, these proteases exhibit a much lower affinity for PAR-1 when compared to thrombin, suggesting that thrombin remains the major mechanism by

which PAR-1 is activated in cells (Camerer et al., 2000; Kuliopulos et al., 1999; Molino et al., 1997; Riewald et al., 2003; Steinhoff et al., 2005).

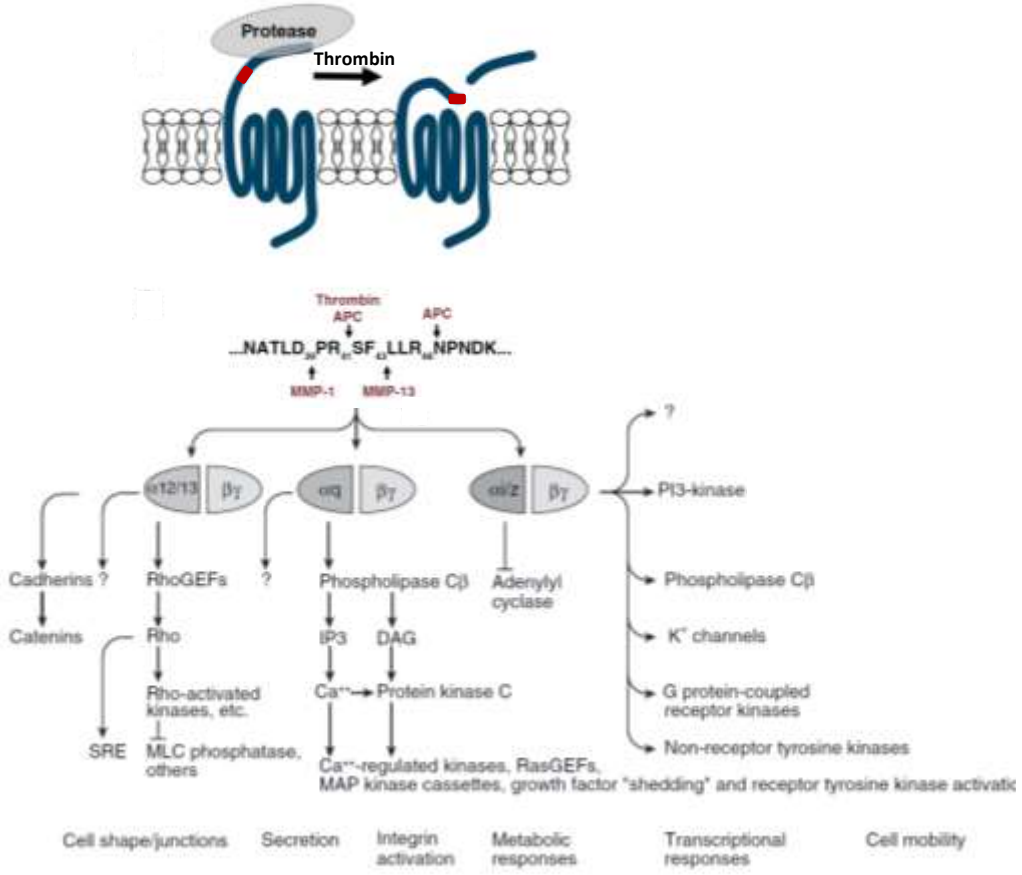

Figure 1.4 PAR-1 activation and downstream signaling. PAR-1 is activated by a proteolytic event at different sites along the N-terminus domain by different proteases including thrombin, MMP1, MMP13, and APC. PAR-1 activates downstream signaling through G proteins and the pathways affect cell shape, mobility and metabolic responses, etc. This figure is adapted from (COUGHLIN, 2005).

Although these proteases exhibit lower affinity or require cofactors to cleave PAR-1, they still retain the capability of competing thrombin, cleaving PAR-1 at different cleavage sites and eliciting distinct downstream signaling events under specific pathological conditions. For instance, the protease APC is about 10,000-fold less potent than thrombin in cleaving PAR-1. However, in cases of sepsis or endotoxemia, APC forms a complex with endothelial cell protein C receptor

(EPCR) on the cell membrane of endothelial cells and cleaves PAR-1 at R46 which is at the C terminus of the thrombin cleavage site. Cleavage of this site creates a biased downstream signaling pathway that is distinct from thrombin-induced activation. The APC-directed cleavage pathway helps to reduce inflammation, stabilize endothelial cell barrier function, and reduces overall mortality (E. Kerschen et al., 2010; E. J. Kerschen et al., 2007; Mosnier et al., 2007; Sinha et al., 2018). Another example is the protease plasmin. As part of the anticoagulant mechanism, plasmin cleaves PAR-1 at R70/K76/K82 sites as a counteract measure to avoid being activated by thrombin. This strategy offers an effective pathway to abolish PAR-1 signaling (Kuliopulos et al., 1999). More recent studies have shown that matrix metalloprotease 1 (MMP1) and MMP13 proteases also activate PAR-1 by cleaving noncanonical sites and inducing distinct downstream cellular effects (Austin et al., 2013; Jaffre et al., 2012; Trivedi et al., 2009) **(Figure 1.4)**. This is particularly important because MMPs are often secreted by tumor cells or cancer associated fibroblasts and thus may contribute to tumor progression via PAR-1 activation in the TME.

PAR-1 has been found overexpressed in many cancer types including breast cancer (Yong-Jun et al., 2003), prostate cancer (Yuan & Lin, 2004), melanoma (Massi et al., 2005), gastric and colorectal cancer (Adams et al., 2015), lung cancer (C. Lin et al., 2017) and pancreatic cancer (Queiroz et al., 2014). However, the mechanism(s) by which PAR-1 affects cancer progression remains cancer type dependent. For instance, MMP1 activates PAR-1 and generates PAR-1 dependent Ca2+ signaling to promote breast cancer cell invasion and tumorigenesis (Boire et al., 2005). In contrast, gastric cancer utilizes thrombin to activate PAR-1, inducing HIF-1 and Twist expression to promote tumor metastasis (Chang et al., 2011). To date, the mechanism by which tumor cell derived PAR-1 is activated in pancreatic cancer is not known. Nonetheless, PAR-1 may be an attractive target for cancer therapeutics. Indeed, over the years, two clinical PAR-1 inhibitors,

Vorapaxar (SCH530348) and Atopaxar (E555), have undergone extensive clinical development. The primary indication of usage of these drugs is for treating thrombotic cardiovascular disease. Despite promising results that inhibition of PAR-1 reduces thrombosis-mediated coronary events, Vorapaxar treated patients exhibited a 5-fold higher risk of internal bleeding complications (reviewed in Moschonas, Goudevenos, and Tselepis 2015).

PAR-2

PAR-2 is the second protease activated receptor that was identified through a mouse genomic library screen and it appears to be the most related PAR to PAR-1 with 30% homology (Nystedt et al., 1994). However, trypsin, instead of thrombin, can cleave between R34 and S35 of its N-terminus and activate this receptor with an EC50 of approximately 1nM (Nystedt et al., 1994). PAR-2 has been found abundantly expressed in the kidney, small intestine, pancreas and stomach (Nystedt et al., 1995). PAR-2 couples and signals with G protein alpha units including Gαq, Gαi, $G\alpha_{12/13.}$ It can also signal via recruiting the adaptor protein β-arrestin to the C-terminus tail in a G protein independent manner (DeFea et al., 2000; Seatter et al., 2004). The interaction between PAR-2 and Gαq or Gαi activates PLC, PKC and MAPK pathways, all of which affect various cellular activities including cell proliferation, morphological changes, cell migration and survival (Berger et al., 2001; Okamoto et al., 2001; Steinhoff et al., 2005). In cancer, PAR2 promotes MDA-MB-231 cell migration through β-arrestin dependent Erk1/2 activation (Ge et al., 2004) and facilitates breast cancer cell chemokinesis through a Src-JNK-paxillin signaling pathway (Su et al., 2009). In hepatocellular carcinoma, PAR-2 promotes cancer cell growth via its expression on hepatic stellate cells, highlighting a stromal PAR-2 function in tumor progression.

PAR-3 and 4

A surprising phenotype of PAR-1 knockout mice was that they exhibited similar tail bleeding times, a measurement of hemostatsis, when compared to wild type mouse. Indeed, platelets from PAR-$1^{-/-}$ mice were still able to aggregate in response to thrombin, indicating that there must be other thrombin responsive receptors (Connolly et al., 1996). Consequently, PAR-3 and PAR-4 were discovered as the second and third thrombin-dependent receptors in the late 1990s. (Ishihara et al., 1997; W. F. Xu et al., 1998). The thrombin cleavage site for PAR-3 is between T39 and F40 and for PAR-4 is between R47 and G48 (Ishihara et al., 1997; W. F. Xu et al., 1998). There are relatively few publications on PAR-3 and PAR-4 downstream signaling cascades. PAR-4 has been shown to interact with $G_{12/13}$ and Gq and activate standard downstream pathways (Faruqi et al., 2000). PAR-3 can also serve as a cofactor for PAR-4 activation by thrombin (Nakanishi-Matsui et al., 2000). In cancer, PAR-4 is overexpressed in colorectal cancer and Trefoil factor 2 promotes colorectal carcinoma cell HT29 invasion via PAR-4 activation (G. Yu et al., 2015). Studies on the role of PAR-3 and PAR-4 in cancer are limited, probably because PAR-3 and PAR-4 are typically expressed at low levels in tumor samples (Elste & Petersen, 2010). Nonetheless, PAR-3 and 4 are thrombin receptors that have the potential to serve as a bypass route if resistance is generated when targeting PAR-1.

1.3 Pancreatic Cancer Microenvironment

1.3.1 *Stromal area composition*

The PDAC stromal area accounts for roughly 80% of the total PDAC tumor volume and is comprised of cancer associated fibroblasts (CAFs), immune cells, endothelial cells, proteins that make up for the extracellular matrix (ECM) and soluble factors such as cytokines, chemokines and growth factors. Despite the multiple and complex components in the stroma, microenvironment

heterogeneity adds yet another layer of complexity to this lethal disease. Stroma composition is not fixed, but rather, it is a dynamic process, changing and evolving along with cancer progression. Brenard et al (2018) harvested PDAC patient cells from early (low grade lesions), medium (high grade lesions) and late stages of PDAC and performed single cell RNA sequencing analysis. Notably, the proinflammatory immune component, including CD8 T cells, T helper cells and dendritic cells, was found in low grade, early stage PDAC, but upon tumor progression, these cell populations are deleted and replaced by myeloid-derived suppressor cells (MDSCs) at late stages (Bernard et al., 2018). A similar heterogeneity is observed with PDAC CAFs where they exhibit different molecular markers that allow them to be categorized as tumor promoting CAFs or immune evading CAFs (Bernard et al., 2018).

Metastasis is the major cause of death in PDAC patients. Recent studies have shown that the PDAC microenvironment related signaling can be a major cause of PDAC cell metastasis. Studies performed using PDAC human autopsy samples revealed a lack of genetic alterations between the PDAC primary tumor and the corresponding metastatic tumors (Haeno et al., 2012; Makohon-Moore et al., 2017). Thus, PDAC metastasis may not be triggered by acquiring additional driver gene mutations as commonly seen in other cancers (Ishaque et al., 2018; Yates et al., 2017). Further genetic analyses in human patient samples and mouse PDAC models have demonstrated that differences between the primary PDAC tumor and the corresponding metastatic lesions involve changes in chromatin structure, metabolic pathways and epigenetic alterations of DNA (McDonald et al., 2017; Roe et al., 2017), highlighting the possibility that extrinsic signaling, rather than intrinsic signaling, promotes PDAC tumor cell metastasis.

1.3.2 *Cancer associated fibroblasts*

Studies have suggested that a robust desmoplastic stromal area contributes to resistance of PDAC to conventional chemotherapy (Farrow et al., 2008; Yen et al., 2002). CAFs represent the major cell population within the stroma, comprising 30% of the cells. There are two sources of CAFs in PDAC; bone marrow derived fibroblasts and resident fibroblasts which are also called pancreatic stellate cells (PSC). PSCs function to maintain normal gland connective tissue. Upon injury or tissue damage, PSCs are activated and differentiate into myofibroblast-like cells, secreting ECM proteins such as laminins, fibronectins and collagens (Apte et al., 1999; Z. Xu et al., 2014). Both *in vivo* and *in vitro* studies have shown that the differentiation of PSCs is triggered by signaling through growth factors and cytokines including endothelin 1, platelet derived growth factor and Trefoil factor 1 (T. Arumugam et al., 2011; Klonowski-Stumpe et al., 2003; Luttenberger et al., 2000; Schneider et al., 2001). After their differentiation, these myofibroblast-like CAFs partner with PDAC tumor cells to support migration and invasion of the cancer cells (Apte et al., 2004; Vonlaufen et al., 2008). For instance, PSC-derived CAFs secrete stroma cell derived factor 1, secreted protein acidic and rich in cysteine (SPARC), and MMPs to facilitate PDAC tumor cell migration and invasion (Koshiba et al., 2000; Mantoni et al., 2008; Schneiderhan et al., 2007). CAFs in PDAC also control ECM protein degradation by producing tissue inhibitor proteases (Wehr et al., 2011), which leads to increased mechanical intratumoral pressure, vascular compression and regional hypoxia. Bone marrow derived CAFs can also supply the resident PSCs and also contribute to fibrosis under inflammation conditions (W.-R. Lin et al., 2012; Scarlett, 2013). Interestingly, Raz et al (Raz et al., 2018) have shown that in breast cancer bone marrow derived fibroblasts have distinct functions and molecular markers that distinguish them from resident fibroblasts. Bone marrow derived fibroblasts express platelet derived growth factor α (PDGFRα) that promotes tumor growth and angiogenesis. In contrast, resident fibroblasts

(PDGFRα negative) mainly regulate ECM remodeling and the recruitment of bone marrow derived cells. This is a particularly important aspect for pancreatic cancer because it provides another possible explanation for the failed SHH inhibitor clinical trials discussed earlier in this chapter where there are conflicting reports regarding the role of CAFs that can both restrict and promote PDAC progression. Lineage tracing studies in the future may help distinguish "good" and "bad" CAFs in PDAC TME and provide us with a more robust therapeutic strategy.

1.3.3 *Immune cells*

PDAC stroma typically contains a prominent leukocytic infiltration that is evident even around low grade precancerous lesions. PDAC stroma is characterized by inclusion of CD4+ T cells, T reg cells, tumor associated macrophages (TAM), a large deposition of myeloid derived suppressor cells (MDSCs) and the absence of CD8+ cytotoxic T cells, which together support a suppressive immune microenvironment (Clark et al., 2007). The suppressive immune microenvironment is supported by studies showing that IL17 produced by CD4+ T cells promotes PDAC immune evasion as inhibition of IL17 in GEM models prevents PDAC initiation even in the presence of oncogenic Kras (McAllister et al., 2014). In addition, PDAC tumor cell derived granulocyte macrophage colony stimulating factor (GM-CSF) is known to recruit myeloid progenitor cells that subsequently differentiate into MDSCs instead of tumor suppressive macrophages. MDSCs later function to prevent CD8+ T cells from clearing transformed tumor cells from the host (Bayne et al., 2012; Srivastava et al., 2010). Interestingly, targeting MDSCs in GEM models leads to increased intratumoral accumulation of activated CD8+ T cells and apoptosis of tumor cells (Stromnes et al., 2014).

TAMs in PDAC have been shown to be pro-tumorigenic and important for tumorigenesis. Embryonically-derived tissue resident TAMS produce the pro-inflammation cytokine IL6, which

activates the STAT3 pathway to accelerate PDAC progression (Lesina et al., 2011; Y. Zhu et al., 2017). Targeting TAMs changes the epigenetic profile of PDAC infiltrated T cells and restores an anti-tumor phenotype (Borgoni et al., 2018). These studies shine a light on the importance of harnessing cells for improved immunotherapy for pancreatic cancer. Given that advances in immune therapies using single-agent immune checkpoint inhibitors targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and the programmed cell death protein 1 (PD-1) were ineffective in PDAC patients (Brahmer et al., 2012; Royal et al., 2010), it might be more productive to employ a combination of PD-1 or CTLA-4 with therapies that can reverse the immunosuppressive microenvironment and thereby provide a better therapeutic outcome.

1.3.4 *ECM proteins*

Collagens are the most abundant and well-studied ECM protein family in PDAC. A total of 28 different collagens have been discovered in PDAC tumors (Ricard-Blum, 2011). In PDAC, collagen I is the major collagen found within the stromal area (Imamura et al., 1995) and deposition of high levels of collagen I correlate with lower patient survival rates (Whatcott et al., 2015). Collagen IV also has been shown to be highly expressed in stromal areas and to promote PDAC tumor cell proliferation and migration (Ohlund et al., 2013).

Hyaluronic acid (HA) and SPARC are also overexpressed as ECM proteins in PDAC. SPARC functions to promote tumor invasion and inhibit angiogenesis (Neuzillet et al., 2013). The collagens and HA in stromal areas contributes to elevated interstitial fluid pressure in the tumor, thereby creating barriers for drug delivery by crushing or compressing blood vessels (Provenzano et al., 2012). HA complexes contribute to interstitial fluid pressure through a process called colloid osmotic effects in which HA functions as a binding core for multiple hydrophilic matrix proteins (Thompson et al., 2010). Enzymatic degradation of HA using PEGPH20 decreases the interstitial

fluid pressure by 84% in mouse models (Thompson et al., 2010). Targeting HA in combination with gemcitabine treatment has shown promising results by increasing drug efficacy in PDAC patients.

The interstitial fluid pressure and compressed blood vessels generated by these ECM proteins creates hypoxic regions within the tumor, which allows tumor cells to not only resist chemo- and radio- therapies but also simultaneously promotes PDAC metastasis (Brown & Giaccia, 1998; Chan & Giaccia, 2007). Chiou et al (Chiou et al., 2017) have discovered a highly metastatic cancer subpopulation in the hypoxia region within PDAC tumors. Further studies have shown that hypoxia can transiently induce transcription factor Blimp1 as an HIF1 target gene. Blimp1 simultaneously halts cell proliferation and promotes cell invasive behavior. This transient process within the hypoxic region emphasizes how the tumor microenvironment guided external signaling can alter PDAC cell behavior.

Taken together, PDAC TME continuously evolves along with cancer cell progression as a dynamic remodeling process that creates a highly immune suppressive, hypovascular and hypoxic microenvironment for cancer cells. However, some individual components of the TME exert both promoting and inhibitory roles for cancer progression. Teasing out the mechanisms by which each component regulates PDAC progression will help us reverse the immune suppressive microenvironment, providing better drug delivery, limiting tumor metastasis and eventually gaining an improved patient prognosis.

1.4 Conclusion

PDAC is a lethal disease characterized by transformed epithelial cells and an abnormal, enriched dysplasia stroma that comprises multiple components and exhibits a complex heterogeneity. Studies to date have established a relatively simple genetics paradigm for pancreatic

cancer where the vast majority of PDAC patients harbor KRas, TP53 and P16 mutations. In principle, with a known cell of origin and driver gene mutations, PDAC progression should be predictable, providing us the opportunity to develop a cure. Genetic analyses of human PDAC samples have helped to reveal that TME external signaling is a potential driver for PDAC progression. Along with abnormal stroma, PDAC patients have the highest rates of thrombotic complications. Targeting thrombotic pathways in patients with cancer has shown promising results and the mechanisms are cancer type dependent. However, patients remain at risk for internal bleeding with these therapeutic strategies. Studies from mouse models have shown encouraging results by targeting key thrombotic factors without disrupting the general coagulation cascade, highlighting the importance of investigating the mechanism(s) operating in each cancer type. These clinical challenges have helped us lay out remaining key questions for pancreatic cancer: (i) what roles do cancer cell derived coagulation factors (TF and PAR-1) and thrombin in the TME play in aggressive PDAC progression?, and (ii) what are the mechanisms by which these factors elicit responses in transformed pancreatic epithelial cells to create PDAC tumors? Studies described in this thesis are designed to address these critical questions.

CHAPTER 2. MATERIALS AND METHODS

2.1 Use of animals ethic statement

Use of animals and animal related experimental procedures were approved by the Purdue University Animal Care and Use Committee (PACUC).

2.2 Cell line isolation and generation

2.2.1 *Acinar cell preparation*

Adult pancreata were obtained from 8-12 week C57Bl/6 mice. Briefly, mice were euthanized, an incision was made along the left lower abdomen side of the mouse and approximately 50 mg of pancreas was cut off from the tail of the pancreas and washed with 1X Hanks' balanced salt solution (HBSS) (Gibco, 14065). The pancreas tissue was placed in a 6 cm dish and injected with 1ml of digestion solution consisting of collagenase P (10 µg/ml) (Roche, SKU-11213857001) and protease inhibitor solution (Pierce protease inhibitor, 1:100, A32965) in 1X HBSS using a 1ml syringe and 27G needle. The tissue was then minced with a microsurgical scissor for 2 mins. The tissue slurry was transferred into a 15 ml Falcon centrifuge tube containing 4 ml of digestion solution to make a total 5ml solution. The tube was then placed horizontally on its side and shaken at 220 rpm, 37℃ for approximately 1 hr. 5 ml of 5% Fetal Bovine serum (FBS) in HBSS solution was added to stop the digestion reaction, followed by gently pipetting up and down 8 times using a 5 ml pipette to dissociate the cell pellet. The cells were then spun at 1,500 g for 3 mins and washed with 5% FBS HBSS solution. Cells were filtered through a 100 µm filter mesh followed by centrifuging for 3 min as above. The cell pellet was subsequently re-suspended in 5 ml of trypsin (0.05% Trypsin EDTA and Pierce protease inhibitor 1:100) HBSS and incubated at room

temperature for 2-5 mins for the generation of individual acinar cells/acini clusters. The cells were then gently washed twice with 5% FBS HBSS solution and processed for RNA isolation.

2.2.2 *KPC cell isolation from tumors*

The mouse KPC cell lines were generated from individual PDAC primary tumors derived from KPC (*Kras*$^{G12D/+}$, *Trp53*$^{R172H/+}$, Elas$^{CreER/+}$) mice. In brief, a single 12-15 mm^3 tumor piece was minced and shaken in digestion solution (1400 U/ml collagenase Type II, Worthington, LS004176 and 0.1 ng/ml Dnase I in 5 ml HBSS) at 220 rpm, room temperature for 1 hr. The dissociated cells were spun down and re-suspended in complete high glucose Dulbecco's modified eagle medium (hgDMEM) containing 10% FBS and 1% penicillin/streptomycin in a 15ml Falcon centrifuge tube. The tube was then placed vertically and left undisturbed for 10 min at room temperature to allow the tumor cells to slowly settle (Note: fibroblasts are more buoyant than tumor cells so it takes considerably longer time for the fibroblasts to settle). The supernatant, which primarily consists of fibroblasts, was carefully removed using a 5 ml pipet. The cell pellet containing tumor cells was then re-suspended in 5 ml of complete Roswell Park Memorial Institute 1640 (RPMI1640) medium containing 10% FBS and 1% penicillin/streptomycin, placed in a 10 cm dish and cultured for at least 4 passages using standard trypsinization protocols prior to characterization.

2.2.3 *shTF and shPar-1 KPC2 cells*

Puro-resistant lentivirus encoding shTF and shPar-1 were obtained from Sigma (TF A: TRCN0000375847; TF B: TRCN0000366885; PAR-1 A: TRCN0000226149; PAR-1 B: TRCN0000226151 shControl vector: SHC001). On day 1, KPC2 cells were placed in a 12-well plate at 200,000 cells per well. On day 2, approximately 10 µl of lentivirus at a 10^7 titer (MOI = 1.0) was applied to each well in RPMI1640 medium supplemented with 8 µg/ml of polybrene

(Sigma, 107689). On day 3, transduced cells were cultured in medium containing 2 µg/ml puromycin (Sigma, P7130) and medium was changed every three days. Approximately 7 days later, puromycin resistant colonies were pooled and used for subsequent experiments.

2.2.4 *PAR-1 and TF CRISPR-generated knockout KPC2 cells*

The dimeric CRISPR RNA-guided Fokl nuclease strategy was used to edit the murine Par-1 gene as described in Tsai et al. (2014). In brief, CRIPSR plasmid pSQT1313 (dual gRNAs plasmid) and pSQT1601 (Cas9 plasmid) were obtained from Addgene (53370 and 53369). Oligos were designed and generated to target the ATG start codon of the PAR-1(*F2r*) and TF (*F3*) genes (see Table 2.1). gRNA oligos were cloned into the pSQT1313 plasmid through the BsmBI site to generate PAR-1 gRNA: pSQT1313 PAR-1 and TF gRNA: pSQT1313 TF. For transfection, 2 x 10^6 KPC2 cells were plated in a 6 cm dish. 2 µg of pSQT1601 plasmid, 250 ng linear puromycin plasmid (Clontech, 631626) and 0.5 µg pSQT1313 PAR-1 or pSQT1313 TF were pre-mixed in serum-free RPMI1640 medium and co-transfected into KPC2 cells using Xtreme Gene 9 (Roche, XTG9-RO). Transfected cells were grown in 2 µg/ml puromycin medium and cultured for 10 days with medium changes every three days. 25 emerged cell colonies were then selected using standard cloning cylinders (Fisher Scientific, 0790710) for screening positive colonies with desired mutations (see section 2.3). At least three clones from each cell line were chosen for further detailed studies.

Table 2.1 CRISPR gRNA plasmid design

CRISPR Primers (5'-3')							
Sequence Name	**Target site1**	**Left dRGN Oligo 1**	**Left dRGN Oligo 2**	**Target site2**	**Right dRGN Oligo 1**	**Right dRGN Oligo 2**	**Notes**
msPAR-1	CCAAG GCTGC CCGCG CGGCC	GCAGC CAAGG CTGCC CGCGC GGCCG TTTTAG	AGCTC TAAAAC GGCCG CGCGG GCAGC CTTGG	CGCTT GCTGA TCGTC GCCCT	GGCAG CGCTT GCTGA TCGTC GCCCT	AAACAG GGCGA CGATC AGCAA GCGC	Spacer=16
msTF	AGGGC TGGAG GCGAG GTCTC	GCAGA GGGCT GGAGG CGAGG TCTCG TTTTAG	AGCTC TAAAAC GAGAC CTCGC CTCCA GCCCT	CGTGC GCCCG CGCCT CCTAG	GGCAG CGTGC GCCCG CGCCT CCTAG	AAACCT AGGAG GCGCG GGCGC ACGC	Spacer=16

2.2.5 *PAR-1$^{KO/Tg}$ cell line*

The PAR-1$^{KO/Tg}$ cell line was generated using KPC2-PAR-1^{KO1} cells. A Tet PAR-1^{myc} plasmid (see section 1.4) was co-transfected with 250 ng linear hygromycin (Clontech 631625) plasmid using Xtreme Gene9 into PAR-1^{KO1} cells. Transfected cells were transferred and grown in 350 µg/ml hygromycin containing medium and medium was changed every three days. 22 cell colonies were selected to screen for doxycycline inducibility of PAR-1. In brief, all colonies were treated with 1 U/ml thrombin for 30 mins and protein from each sample was harvested followed by immunoblotting for p-Erk. A total of 10 out of 22 colonies successfully restored Erk activation upon thrombin treatment. Three colonies were kept for further studies.

2.3 PAGE gel CRISPR screening

A PAGE gel CRISPR screening protocol was modified from X. Zhu et al. (2014). In brief, DNA was isolated from the parental KPC2 line as well as from candidate KPC2-PAR-1^{KO} and KPC2-TF^{KO} clones. Standard PCR was performed using primers flanking the targeted mutation area of PAR-1 and TF. 4 µl of WT KPC2 PCR product and 16 ul of KPC2-PAR-1^{KO} or KPC2-TF^{KO} PCR product were mixed, heated to 95℃ and then slow cooled to room temperature to allow products to anneal. The material was then loaded onto a 15% non-denaturing polyacrylamide gel (PAGE) and electrophoresed at 90v for 90 mins to visualize potential heteroduplexes (**Figure 2.1 A&B**). Potential mutated DNA PCR products were isolated from the PAGE gel using a standard DNA extraction process.

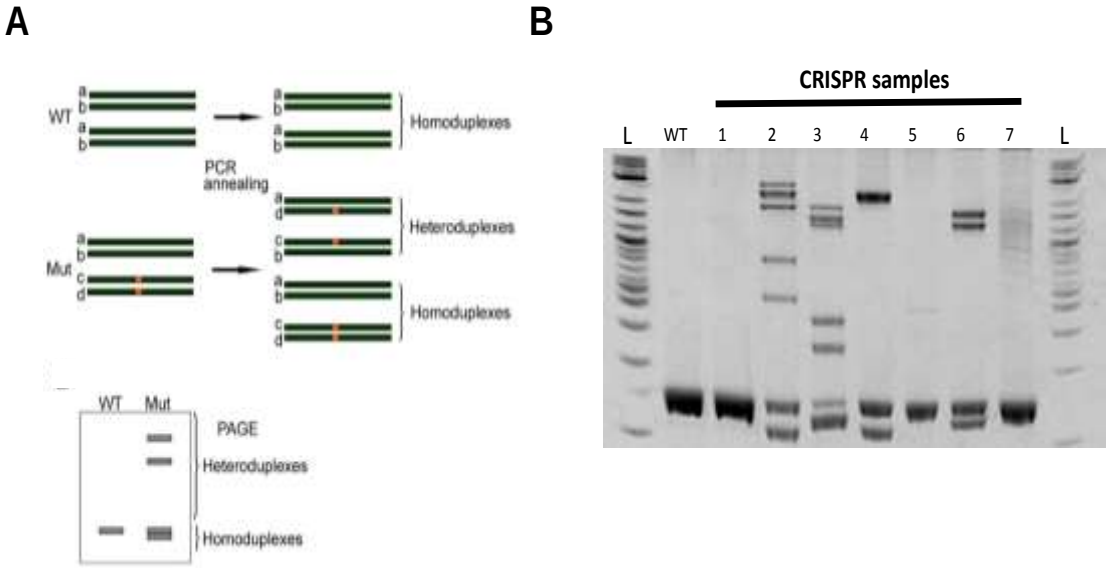

Figure 2.1 PAGE gel CRISPR screening. A. Basic principle of PAGE gel CRIPSR screening. **B.** Sample results from PAGE gel screening. Sample 2, 3, 4 and 6 have DNA mutations.

Table 2.2 CRISPR screening primers

CRIPSR Screening Primers (5'-3')

	Primer left	Primer right
msPAR-1_S	CCCTGAACCGCCTCCAAATA	GCTCCTGGGGGAAACTGAGGCAG
msTF_S	CCCATCACTCGCTCCCTCCGATC	CCCAAGGAACCAGAGCAGCACCC

2.4 Plasmid construction and cell transfection

The mouse PAR-1 open reading frame was obtained through reverse transcription PCR of KPC2 cell mRNA followed by cloning into the pcDNA 3.1 plasmid to attach the Myc epitope tag at the PAR-1 cDNA C-terminus. The PAR-1^{Myc} sequence was then sub-cloned into the Tet-one plasmid (Clontech, 634301). Doxycycline inducibility of the plasmid was first tested by transfecting Tet-one PAR-1^{Myc} into 293T cells and Myc tag expression was confirmed by immunofluorescence. For cell transfections, plasmids were introduced into cells using Xtreme Gene 9. In brief, 7.5 µl Xtreme Gene 9 transfection reagent and 2.5 µg plasmid was pre-mixed at room temperature for 20 mins in 100 µl serum-free medium. The transfection mix was then added dropwise to the cells in a 6 cm dish with complete RPMI1640 medium. Transfected cells were harvested for screening or analyzed at 48 hrs post-transfection.

2.5 Subcutaneous or orthotopic tumor growth and lung metastasis assays

Subcutaneous tumor studies: KPC2 cells and derivative cell lines were trypsinized, counted using a hemocytometer, spun down and re-suspended in cold sterile PBS. 8-12 week C57Bl6 mice were anesthetized before injection. Cells in cold PBS were injected into the intrascapular region using a 1ml syringe and 27G needle containing cells at a concentration of 2.5×10^5 in 100 µl sterile PBS. After injection, mice were placed on a heating pad for 5 mins to recover from anesthetize. Tumors were measured using a scientific caliper over time starting at day 8 post-injection and at every 2-3 days until the study was terminated. Tumor volume was calculated (Tomayko &

Reynolds, 1989) as Volume = (Length x Width^2)/2. Tumor mass was weighed on the last day of harvest.

Orthotopic studies: All surgical tools were autoclaved and placed under the tissue culture hood before use. 8-12 week mice were anesthetized using isoflurane prior to surgery. The left side abdomen was shaved and cut open using surgical scissors. The pancreas was identified by first locating the red colored spleen on the left side of the body cavity. The organ was then gently pulled out of the incision and exposed for injection. Cells in cold PBS were injected into the tail of the pancreas with the needle towards the head of the pancreas at a concentration of 5 x 10^4 cells in 20 µl sterile PBS using a 0.30cc syringe and 30G needle. The pancreas was then gently placed back into the body cavity and the surgery wound was sutured and iodine was applied to prevent infection. 50 µl of 0.03 mg/ml buprenorphine (Buprenex Injection, NDC 12496-07575) was injected subcutaneously into each mouse to relieve any post-surgery pain. Mice were then placed on a heating pad for 5 mins to aid in recovery.

In experiments in which prothrombin levels were reduced pharmacologically, hepatic prothrombin synthesis was suppressed using a 20-mer antisense oligonucleotide (ASO) "gapmer" complementary to a portion of the 3′ noncoding region of the mouse prothrombin mRNA. C57Bl/6 mice received weekly intraperitoneal injections of 50 mg/kg prothrombin ASO (5′-attccatagtgtaggtcctt-3′) in 200 µl of sterile PBS or a control 20-mer (5′-ccttccctgaaggttcctcc-3′) gapmer for a total of 3 weeks prior to subcutaneous tumor cell injection (Horowitz et al., 2011). The weekly ASO treatment was maintained during the course of tumor growth. In the orthotopic experiment, mice were treated with ASO 3 days post tumor cell implantation. In the experiments of activating Tet-PAR-1 expression *in vivo*, mice were provided doxycycline chow (ENVIGO, TF 08541) with food replaced every 6 days.

For experimental metastasis assays, 8-12 week mice were anesthetized before injection. Heated lamp and ethanol were applied to the tail in order to visualize the tail vein. 5 x 10^4 cells of each cell line in 200 µl cold PBS were administered by tail vein injection using a 1ml syringe with 27G needle. Three weeks after injection the lung tissue was harvested, weighed, and analyzed by histology.

2.6 *In vitro* doxycycline and thrombin treatment

KPC2 cells or KPC2 derivative cells were plated at 60% confluence in a 6 cm dish in growth media. On the following day, cell media was removed and 3 ml of RPMI1640 medium ± 1 µg/ml doxycycline hyclate (Sigma, D3447) supplemented with 1% FBS and 1% penicillin/streptomycin was added for 24 hrs. 1 µl of 3 U/µl thrombin stock was added evenly on the cells to make the final thrombin concentration 1 U/ml (Enzyme Research Lab, BT-1002a). Cells were incubated ± thrombin for 1 hour at 37℃, 5% CO_2 and then cultures were harvested for immunofluorescence, immunoblotting, or RT-qPCR analyses.

2.7 Immunoblotting

Immunoblotting was performed as previously described (Karki et al., 2015). In brief, protein lysate was harvested using standard RIPA buffer containing sodium vanadate (Sigma, 450243), protease inhibitor solution and phosphatase inhibitor cocktails 2 and 3 (Roche P5726, P0044) followed by sonication using a Vibra Cell Sonicator. The protein concentration of each sample was measured using the Bio-Rad protein assay (Cat: 5000006). 30~60 µg protein from each sample was mixed with Laemmli sample loading buffer (Bio-Rad, 1610747) and loaded onto 10~12% SDS polyacrylamide gels (Bio-Rad, 1610154). Electrophoresis was performed at 90v for 90 mins.

Protein samples were then transferred to PVDF membranes (BioRad 1620177). Membranes were blocked in 5% milk Tris-buffered saline (TBST) for 1 hr at room temperature followed by probing with primary antibodies for p-Erk 1:1000 (Cell Signaling, 9102), total Erk 1:1000 (Cell Signaling, 4377s), and Hsp90 1:5000 (Santa Cruz, SC-7947) for 1 hr at room temperature. The membranes were then washed with TBST buffer three times for 10 mins each time, followed by incubation of a HRP-conjugated secondary antibody (1:5000, Vector Laboratories, PI3000&PI1000) for 1 hr in TBST buffer. ECL (Thermo Scientific 32106) reagents were applied to membranes for visualizing the protein band under the Bio-Rad ChemiDoc Touch Imgaing System.

2.8 Histology, immunohistochemistry and immunofluorescence staining

Pancreas tissue or PDAC tumors were fixed with 10% neutral buffered formalin solution overnight followed by embeding in paraffin. Sections were generated by using standard histological techniques and individual tissue sections were deparaffinized and antigens were retrieved using a 2100-retriever (Electron Microscopy Sciences, 62700-10) and antigen unmasking solution (Vector Laboratories, H3301). For immunohistochemistry staining, sections were first treated with 3% H_2O_2 for 5 mins at room temperature to eliminate the endogenous peroxidase activity, followed by immunogen blocking using the M.O.M. blocking reagent (Vector Laboratories, BMK 2202). Primary antibodies were then applied to the slides for 1 hour at room temperature for TF (1:200, Abcam, 151748) and K19 (1:100, Dev. Studies Hybridoma Bank, troma3) or 4^{o}C overnight for PAR-1 (1:100, BD Transduction Laboratories 611523), phospho-H3 (1:1000, Millipore sigma 06570), cleaved caspase-3 (1:1000, Cell Signaling, D175), IbaI (1:1000, FUJIFILM Wako Pure Chemical Corporation, 016-20001), and CD3 (1:100, Abcam 16669). Biotinylated secondary antibody (Vector Laboratories, BA9200) was applied to tissue sections at 1:200 dilution for 10 mins at room temperature followed by treating with Vector developing

reagents and DAB (diamonibenzidine) peroxidase substrate (Vector Laboratories, SK4105, PK7100). For immunofluorescence staining, anti-Myc (1:500, Santa Cruz, SC-40) and avidin-conjugated Alexa Fluor 594 (1:100, Invitrogen, S11227) were used. For Hematoxylin and Eosin staining, histological section slides were treated with hematoxylin (Lerner laboratories, 1931382) for 2 mins after the deparaffinization procedure, followed by washing in tap water for 30 dips. Stained slides were then treated with 0.25% Ammonia water (Electron Microscopy Sciences, 2612310) for 30s followed by incubation in Eosin solution (Lerner laboratories, 1931492) for 2 mins. The slides were subsequently dehydrated and mounted using mounting medium (Vector Laboratories, H5000).

2.9 RT-qPCR

Table 2.3 RT-qPCR primers

Gene	Primer left (5'-3')	Primer right (5'-3')
F2r	CCAGCCAGAATCAGAGAGGAC	AGTAGACTGCCCTACCCTCC
F2rl1	CTGGGAGGTATCACCCTTCTG	GAGAACTCATCGATGGAAAAGC
F2rl2	TTTCCACTTGCTGCTCATACAT	AGTTAAGGTTGGCTTTGCTGAG
F2rl3	CTGCTGTATCCTTTCGTGCTG	ACTGTCGTTGGCACAGAATTT
F3	ACAATTTTGGAGTGGCAACC	TCACGATCTCGTCTGTGAGG
Rn18s	TGTCTCAAAGATTAAGCCATGC	GCGACCAAAGGAACCATAAC

Total cellular RNA was extracted and harvested using the E.Z.N.A. Total RNA kit (OMEGA, R6834-02). For quantitative RT-qPCR, 1 µg of each RNA sample was first converted to cDNA using the iScript cDNA synthesis kit (Bio-Red, 1708891). The reaction protocol was 25℃ for 5mins for priming, 46℃ for 20 mins for the reverse transcription reaction and 95℃ for 1 min to stop the reaction. Quantitative PCR was set up with the following formula: 2 µl of cDNA, 1 µl of 10 µM gene primer mix, 10 µl SYBR green 2X master mix (Roche, 04913850001) and 7 µl ddH2O.

Quantitative PCR was performed in duplicates in 96-well plates using a Roche LightCycler 96 thermocycler. The thermal cycling parameters were as follow: 95℃ for 10s, 59℃ for 10s, 72℃ for 10s for 40 cycles. All transcript levels were normalized to internal control 18S expression using the ΔΔCT method to determine the relative fold change. Primer sequences for mouse PAR and TF transcripts are as follows:

2.10 Cell proliferation assays

Cell growth rates were determined by the CyQUANT cell proliferation assay following manufacturer's instructions (Thermo Fisher, C7026). Briefly, 500 cells of each cell line were cultured in four 96-well plates with 4 technical repeats per plate. The cells were culture in 5% CO_2 at 37℃ for 24 hrs. One plate (24 hrs timepoint) was subsequently washed with 1X PBS followed by a snap freeze at -80℃. Plates were continually rinsed and frozen at 48 hrs, 72 hrs and 96 hrs. All time point plates were thawed before reading at room temperature. CyQUANT lysis buffer with fluorescence dye was added to the plates and incubated at room temperature for 5 mins. Plates were loaded onto a fluorescence plate reader and fluorescence intensity of each well was measured with excitation of 480nm and emission of 520nm. Data were colleted and plotted using Graphpad Prism 7 software. Doubling time was calculated as Duration x log(2)/log(final fluorescence) - log(initial fluorescence) using the log phase time points.

2.11 Soft agar assay

Two layers of soft agar were applied in a 6 well plate. The bottom agar layer was used to support the top agar layer containing tumor cells. First, 1.5 ml of 0.6% bottom layer agar (DIFCO,

214010) in RPMI1640 complete medium was placed at the bottom of the plate. After the bottom layer agar solidified, 1000 cells in 1.5 ml 0.4% agar in RPMI1640 medium was subsequently applied on top of the bottom agar layer. Once the agar solidified, 1 ml of RPMI1640 medium was added to each well for nutrition and the medium was changed every 5 days. The 6 well agar plate was cultured at 37℃, 5% CO_2 for 21 days followed by staining with 0.5 ml of 0.1% Crystal Violet in 10% ethanol for 30 mins at room temperature. Each well was then washed with 1X PBS for at least three times until the blue color in the agar dissipated. Colony number was counted using ImageJ.

2.12 DNA extraction

Approximately 10^6 KPC2 cells or PAR-1 knockout cells were counted and spun down by centrifugation in a 1.5 ml centrifuge tube. 500 µl of Lysis buffer (100 mM Tris-HCl, 10 mM EDTA, 200 mM NaCl and 0.2% SDS, pH:8.5) and 10 µl of 10 mg/ml protease K was applied to the cells and incubated at 56℃ overnight. The cell lysates were then spun down at 14,000 rpm for 10 mins followed by transferring the lysate to a new 1.5 ml tube. 500 µl of isopropyl alcohol (MACRON fine chemicals, 303216) was added to the tube and incubated at -20℃ for 20 mins to increase DNA participation. The samples were then quickly spun down at 14,000 rpm for 10 mins. The supernatant was discarded and the DNA pellet was washed with 70% ethanol. The washed DNA pellet was then resuspended in 150 µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH: 8.0) for further analysis.

2.13 Statistics

RT-qPCR, tumor mass, and lung mass data were analyzed by unpaired Student's *t*-test. Cell growth doubling time and colony numbers were analyzed using One-way ANOVA. Tumor volumes and cell numbers over time data were analyzed by repeated measures ANOVA. All data are presented as the mean ± SEM. The statistics of mouse survival was calculated by the Kaplan-Meier Log Rank analysis with Chi square = 26.76. * $P < 0.05$; ** $P < 0.01$; ***: $P < 0.001$; n.s.: not significant.

CHAPTER 3. BOTH CANCER CELL DERIVED TISSUE FACTOR AND THROMBIN IN THE TUMOR MICROENVIRONMENT PROMOTE PDAC GROWTH AND METASTASIS

3.1 Introduction

Tissue Factor, a 43 kDa glycoprotein found on the cell membrane, serves as the initiator of the coagulation cascade. However, TF can also be secreted by being anchored on microparticles (MPs). A microparticle is one of three different membrane vesicles that include exosomes (50 – 100 nm in diameter), MPs (100 nm – 1 μm in diameter) and apoptotic bodies (1μm – 5 μm in diameter), all of which are released by the cell (Gyorgy et al., 2011). The ability of TF to associate with MPs makes it easy to have TF infiltrate the TME, activate prothrombin cleavage and initiate the coagulation cascade. Indeed, TF bearing microparticles derived from tumor cells cause coagulation activation *in vivo* (Davila et al., 2008). In PDAC patients, abnormal thrombosis is one of the most common cancer complications due to robust activation of coagulation cascade in the PDAC TME, and indeed, it is well established that *F3* (TF gene) expression by tumor cells serves as a critical link between cancer and cancer-associated thrombosis (van den Berg et al., 2012).

The assumption that the pathological role of the TF-thrombin axis in cancer is limited to thrombosis has been replaced by the belief that TF, thrombin, and downstream targets also promote cancer progression (Palumbo, 2008). TF levels correlate with disease histological grade, and high TF expression in tumor specimens is an important negative predictor of PDAC patient survival (Kakkar et al., 1995; Nitori et al., 2005). Additionally, thrombin has the capacity to drive multiple aspects of tumor biology, such as cleaving fibrinogen and forming a fibrin clot microenvironment to support tumor growth and angiogenesis (Adams et al., 2015). In addition, pharmacological reduction of thrombin limits colon cancer severity and inhibitors of thrombin have been shown to

block metastasis of various cancer cell lines (*e.g.*, fibrosarcomas, lung carcinomas) (Adams et al., 2015; Bobek & Kovarik, 2004; Esumi et al., 1991). However, the role of the TF-thrombin axis (**Figure 3.1**) in PDAC has not been previously tested. In this study, I have examined TF levels in patient and mouse PDAC samples. I also have investigated tumor growth and metastasis properties of mouse PDAC derived cells upon inhibition of TF in tumor cells as well as by using genetic and pharmacological strategies to inhibit thrombin in the TME.

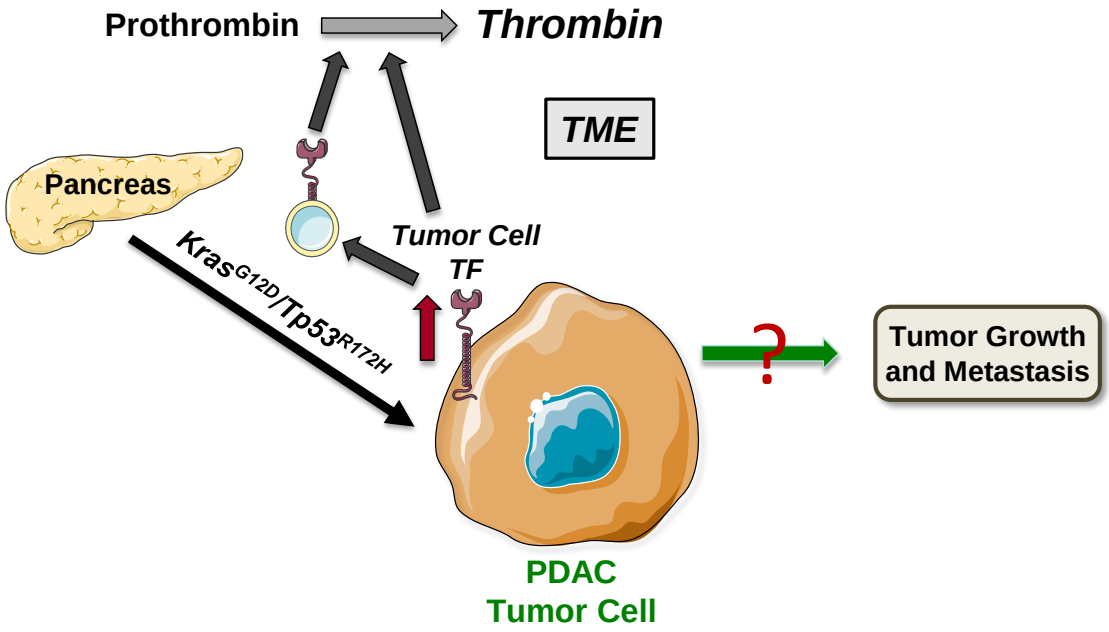

Figure 3.1 Model of the current working hypothesis. Transformed pancreatic epithelial cells upregulate TF expression, creating a robust thrombotic TME to support cancer cell growth and metastasis.

3.2 Results

3.2.1 *Tissue factor expression by transformed cells contributes to PDAC tumor growth*

In order to investigate if TF plays a role in PDAC disease, I first examined TF levels in PDAC patient samples. Analysis of a number of independent human PDAC tumors revealed high TF protein levels in epithelial cells of early PanIN lesions and mature PDAC tumors, whereas TF

protein was not detected in acinar or duct cells of histologically normal pancreas tissue (**Figure 3.2A**). To determine if the same TF expression pattern is reproduced in a mouse PDAC model, we examined TF protein levels in pancreas tissue obtained from genetically modified 'KPC' mice carrying acinar-specific inducible alleles encoding active K-Ras and dominant negative Trp53 ($\underline{K}ras^{G12D/+}$, $Trp\underline{5}3^{R172H/+}$, $Elas^{\underline{C}reER/+}$). Importantly, KPC mice develop PDAC disease that readily mimics the genetic, biochemical, immunological and histological features of PDAC in patients (Habbe et al., 2008; Hingorani et al., 2003; Shi et al., 2009). Similar to PDAC patient tissue, robust TF staining was observed in epithelial cells of mouse PanIN and PDAC lesions but was absent in WT pancreas cells (**Figure 3.2B**). As predicted, high *F3* transcript levels (TF gene) were also detected in KPC-derived PanIN and PDAC tissues with little to no detection of *F3* transcripts in normal WT pancreas or in isolated WT acinar cells (Ac) (**Figure 3.2C**).

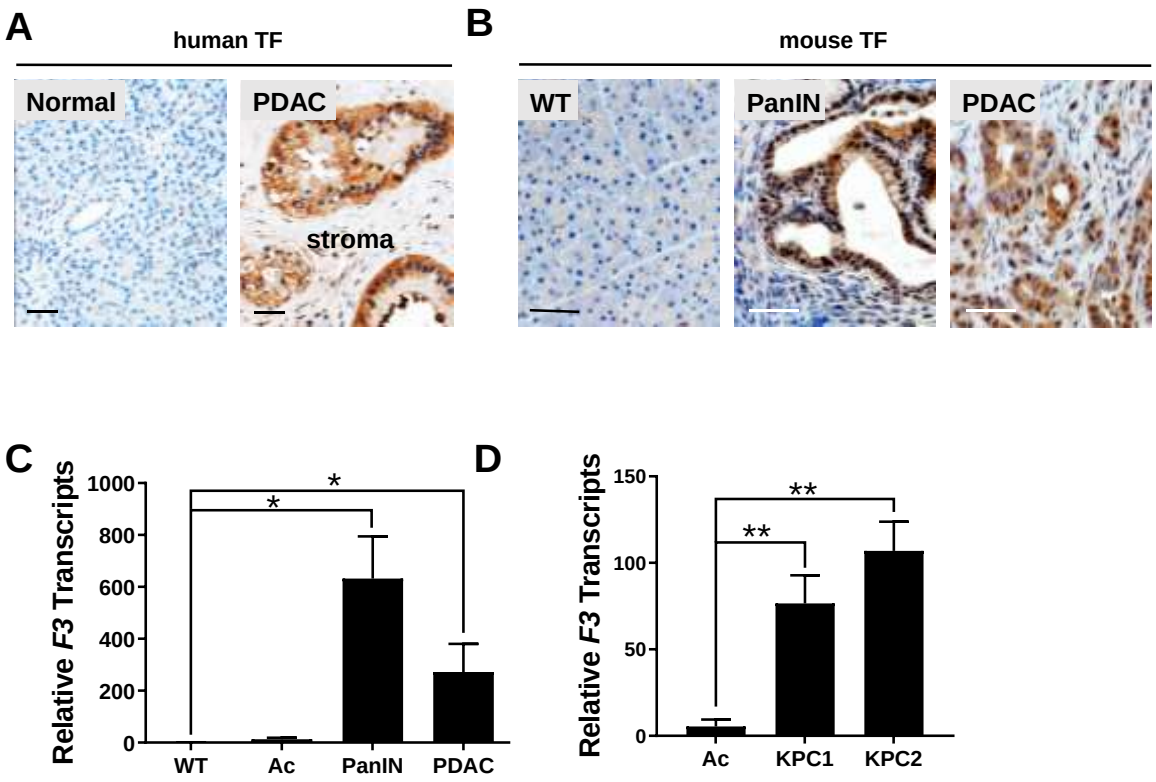

Figure 3.2 Tissue Factor (TF) expression in PDAC. (**A**) Human TF immunohistochemistry (brown) in normal pancreas and in PDAC patient samples. (**B**) Mouse TF immunohistochemistry in normal pancreas (WT) as well as in PanIN lesions and PDAC tumors in the KPC model. (**C**) RT-qPCR analysis of mouse *F3* transcripts in WT pancreas, isolated acinar cells (Ac), PanIN, and PDAC tissue (N=3 per group). (**D**) RT-qPCR analysis of *F3* transcripts in isolated Ac preparations and in the mouse PDAC tumor cell lines KPC1 and KPC2 (N=3 per group). * $P < 0.05$; ** $P < 0.01$.

To determine if TF expression by $KRas^{G12D}/Trp53^{R172H}$ transformed cells plays a functional role in PDAC disease progression, we next generated KPC-derived tumor cell lines (termed KPC1, KPC2) from KPC mice. As shown in **Figure 3.2D**, high TF gene expression was maintained in independent KPC tumor cell lines when compared to normal pancreas acinar cells.

Given the observed elevated levels of TF in PDAC disease, I decided to pursue a genetic approach to analyze TF function and its potential importance in PDAC progression. As a first step, stable TF-specific shRNA knockdown KPC2 lines were generated using targeted shRNA lentiviruses. Whereas control shRNAs showed no change in *F3* expression, shTF cell lines

exhibited an approximate 90% reduction in *F3* transcripts (**Figure 3.3A**). Notably, KPC2 shControl cells and shTF cells were morphologically indistinguishable and had virtually identical proliferation rates (**Figure 3.3B, C**), suggesting that knocking down TF did not alter intrinsic cell signaling *in vitro*. In contrast, subcutaneous tumor growth in immunocompetent C57Bl/6 mice revealed a dramatic decrease in tumor volume over time for the KPC2 shTF cells when compared to the KPC2 shControl cells (**Figure 3.4A)**, resulting in a 2.5-fold reduction in final tumor mass (**Figure 3.4B**). Together, these results suggest that TF expression is critical in promoting PDAC tumor progression. The stark differences in the effect of TF silencing between *in vivo* and *in vitro* PDAC growth potential also suggest that TF promotes tumor growth through mechanisms that are dependent on the host microenvironment.

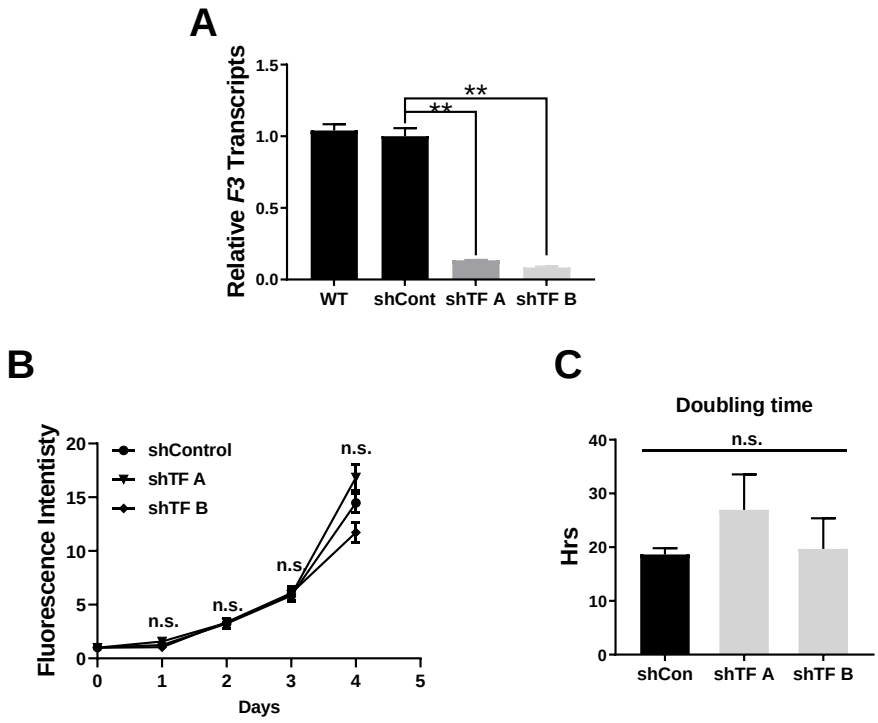

Figure 3.3 Knock down TF in KPC2 cells does not alter cell proliferation *in vitro*. (**A**) RT-qPCR of *F3* transcripts in KPC2 WT, KPC2 shControl and KPC2 shTF 'knock down' cell lines. shTF A and shTF B represent two different shRNA sequences. (**B&C**) Growth curve and doubling times for KPC2 WT, KPC2 shCont and shTF cell lines in culture. ** $P < 0.01$. n.s.: not significant.

To determine if TF contributes to PDAC dissemination, we decided to determine if KPC cells could metastasize to lungs when injected into the mouse tail vein. These studies were performed in collaboration with Dr. Matthew Flick at the Cincinatti Children's Hospital Medical Center (CCHMC), where Dr. Flick performed the actual injections. As shown in **Figure 3.4C, D**, KPC2 shControl cells generated substantial metastatic burden in lung tissues 3 weeks post-injection such that individual metastatic lesions were too numerous to quantify. In contrast, KPC2 shTF cells produced markedly fewer distinct metastatic foci (**Figure 3.4C**). Lung weight analyses revealed a significantly higher lung mass for mice injected with KPC2 shControl cells owing to the extensive tumor burden in these samples versus lungs harvested from mice injected with shTF cells (**Figure 3.4D**). Standard H&E histology confirmed KPC2 shControl tumor tissue throughout the organ, effectively replacing lung epithelial cells and parenchyma. In contrast, mice injected with the shTF cells exhibited only a few distinct metastatic foci with a large portion of lung architecture remaining unperturbed (**Figure 3.4C**). There are several possibilities that may explain these results, including; i) shTF cells exhibit less adhesion capability so they do not efficiently adhere to the lung; ii) shTF cells successfully migrate to the lung, but the microenvironment in the lung inhibits growth, or iii) shTF cells initially seed and grow in the lung but, the environmental stress in the lung (e.g., immune system), induces substantial cancer cell death when compared to the shControl cells. Further investigation is needed to elucidate the detailed mechanism downstream of TF that accounts for the poor metastatic properties of KPC2-shTF cells.

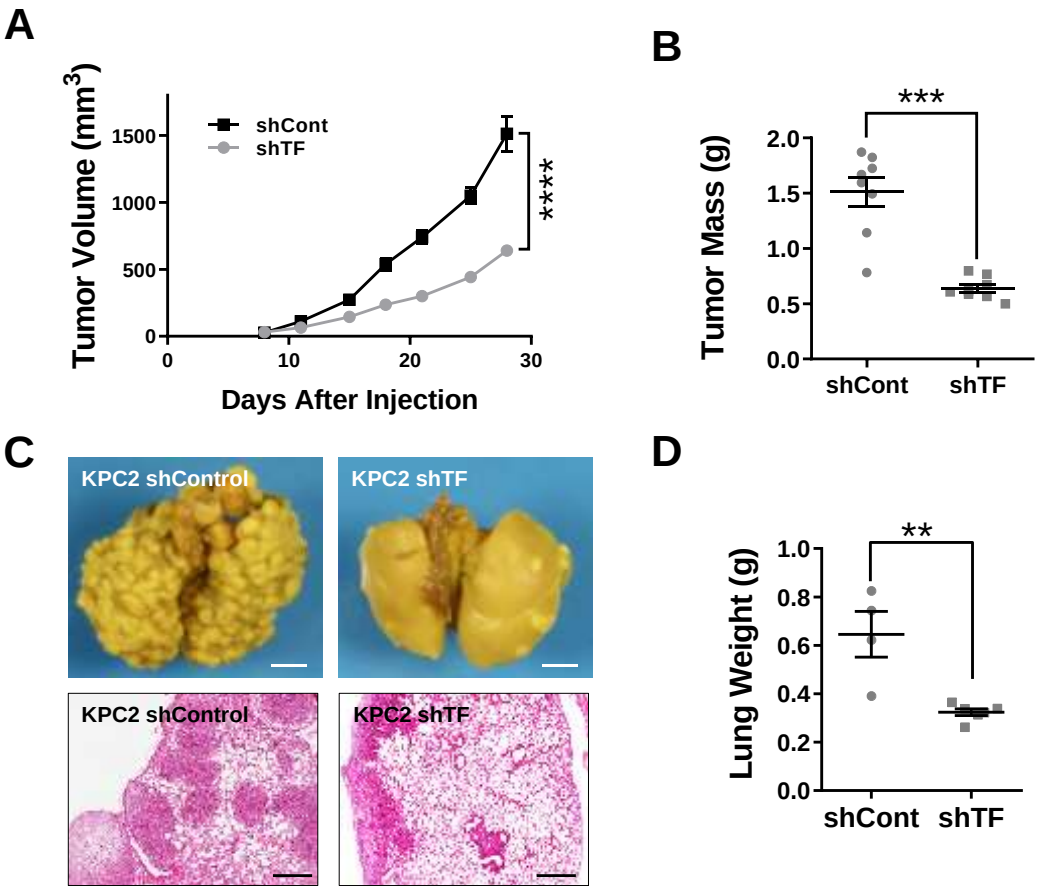

Figure 3.4 Cancer cell derived TF expression contributes to PDAC tumor growth and metastasis. (**A, B**) Analysis of tumor growth following subcutaneous injection of KPC2 shControl and KPC2 shTF lines in WT mice (N=8 mice per group). Tumor volume was measured over time and tumor mass quantified at 4 weeks of growth. **(C, D)** Analysis of lung metastasis following tail vein injections of KPC2 shControl and KPC2 shTF cells (N=4-6 mice per group). Representative images of lungs harvested 21 days post-injection, total lung weight, and H&E stained lung sections. * $P < 0.05$; ** $P < 0.01$; ***: $P < 0.001$. Scar bar: 2 mm for the lung pictures, 0.5 mm for the H&E. These studies were performed in collaboration with Dr. Flick at CCHMC.

3.2.2 *Prothrombin promotes PDAC tumor growth and metastasis*

We hypothesized that cancer cell-derived TF primarily promotes PDAC tumor growth by driving activation of the extracellular serine protease thrombin found within the TME. To test our hypothesis, I evaluated tumor growth of injected KPC2 cells in genetically modified mice (termed *fII*^{low}) that express 10% of normal circulating prothrombin levels due to a hypomorph mutation in the prothrombin gene (Mullins et al., 2009). Interestingly, KPC2 tumors were significantly smaller in *fII*^{low} mice when compared to WT mice, revealing an important role of TME prothrombin in supporting tumor growth (**Figure 3.5A, B**). As a complementary approach to using *fII*^{low} mice, we turned to a pharmacological strategy to reduce prothrombin levels. Briefly, we utilized a highly selective murine prothrombin anti-sense oligonucleotide (ASO) "gapmer" that lowers circulating prothrombin levels to approximately 5% of normal (P. I. Arumugam et al., 2015; Crosby et al., 2015). For this approach, mice were injected weekly with either control ASO or prothrombin ASO (50mg/Kg) via intraperitoneal injection and tumor volume was measure over time. As expected, a control ASO had no effect on KPC2 tumor growth whereas lowering prothrombin levels with the prothrombin ASO significantly impeded KPC2 WT tumor growth, resulting in a reduced final tumor mass (**Figure 3.5C, D**). Next I asked if reduction of prothrombin could similarly limit tumor growth of KPC cells in the pancreas by performing orthotopic injections of KPC2 cells. For these studies, mice were anesthetized and surgery was performed to make a single excision to locate the pancreas. The pancreas was carefully exposed and KPC2 cells were injected at the tail of pancreas. ASO was administrated 3 days post-surgery to test the therapeutic effects of inhibiting prothrombin after the tumor established in the pancreas. As shown in **Figure 3.6A**, circulating prothrombin was reduced to non-detectable levels in mouse plasma by pharmacological treatment with prothrombin ASO. After injection of the tumor cells, ASO treated mice exhibited significantly smaller KPC2 tumors at 21 days (**Figure 3.6B, C**). Thus, genetically (*fII*^{low}) or pharmacologically (ASO) reducing

prothrombin levels significantly diminishes KPC tumor growth, even in the context in which prothrombin reduction occurs following the seeding of tumor cells within the pancreas. These results support a significant role of TME localized thrombin in PDAC disease.

To determine if circulating prothrombin can also promote PDAC metastasis, KPC cells were tested in tail vein injection metastasis assays using *fII*^{low} mice as described earlier. A significant reduction in lung metastatic potential was observed when KPC2 WT cells were injected into *fII*^{low} mice vs. WT mice (**Figure 3.7A, B**), a phenotype mimicking our results obtained with the shTF KPC2 cells (**Figure 3.4**). One possibility for the reduced metastatic potential of KPC2 in *fII*^{low} mice is that KPC2 cells might exhibit a decrease in cell migration or in the initial adhesion of the cells to the lung. In order to test this hypothesis, I generated KPC2^{GFP} cells and examined lungs for the presence of GFP+ cells 24 hours post-injection. As shown in **Figure 3.7C, D**, KPC2^{GFP} micro-metastases were readily observed in WT mice. In contrast, KPC2^{GFP} cells failed to significantly accumulate in lungs of *fII*^{low} mice 24 hours post-injection. Taken together, these results suggest that KPC2 cells fail to migrate to the lung and survive in low prothrombin mice (**Figure 3.7C, D**).

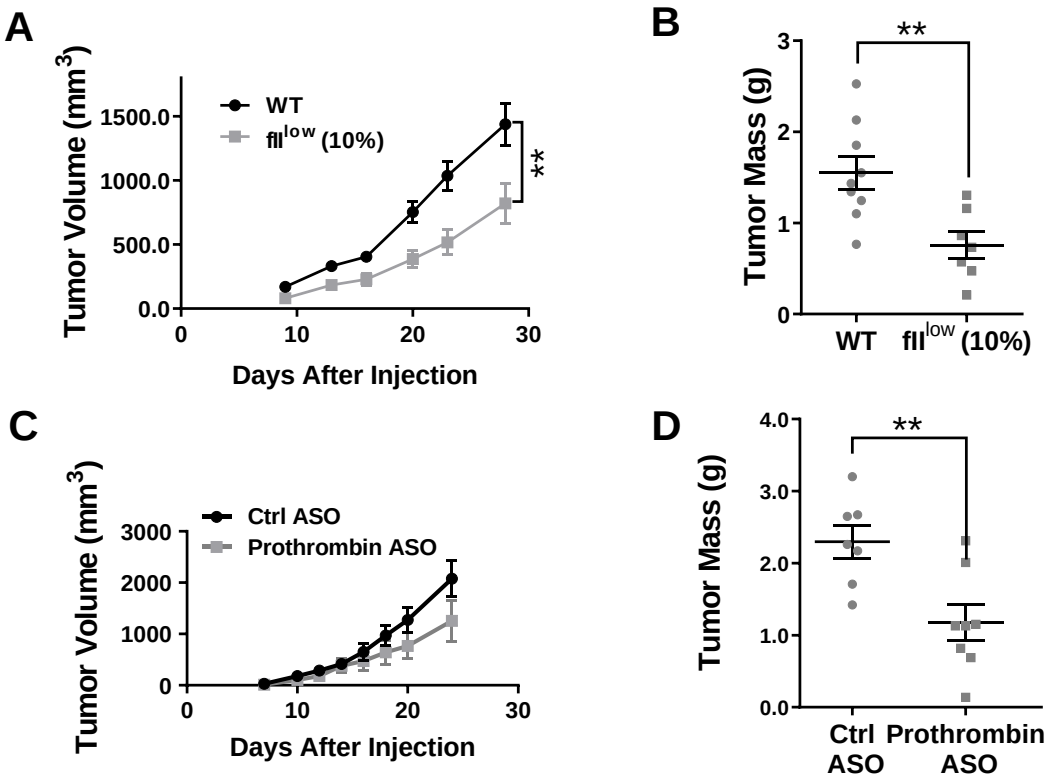

Figure 3.5 Prothrombin promotes PDAC tumor growth. (**A,B**) Analysis of tumor growth following subcutaneous injection of KPC2 WT cells into C57Bl/6 WT or *fII*^{low} mice (N=7-8 mice per group). Tumor volume was measured over time and tumor mass established for isolated tumors following 4 weeks of growth. (**C,D**) WT C57Bl/6 mice were administered a prothrombin specific antisense oligonucleotide (ASO) gapmer or a non-targeting control gapmer (N=8 mice per group) and analyzed for KPC2 cell tumor growth following subcutaneous injections. Tumor volumes were measured over time and tumor mass was determined for isolated tumors following 4 weeks of growth. * $P < 0.05$; ** $P < 0.01$. These experiments were performed in collaboration with Dr. Flick.

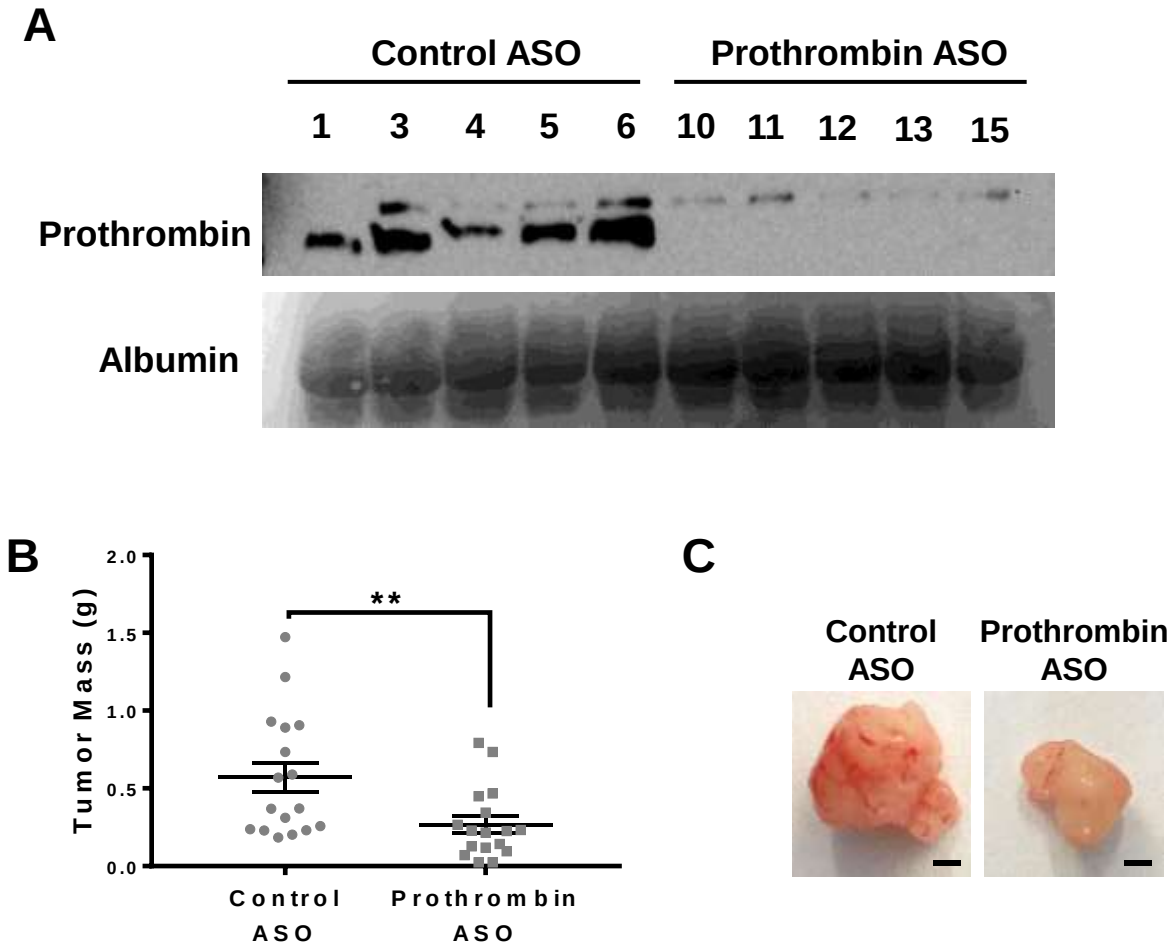

Figure 3.6 Inhibition of prothrombin impairs KPC2 tumor growth in the pancreas. (A) Immunobloting analysis of prothrombin levels in mouse plasma from Control ASO and Prothrombin ASO treated groups. Albumin was used as a loading control. This immunoblot was performed by Dr. Flick. **(B,C)** Analysis of the final tumor mass 21 days following orthotopic injection of KPC2 cells into the pancreas of C57Bl/6 mice treated with a control or prothrombin-specific ASO weekly following injection of the tumor cells. ** $P < 0.01$. Scar bar: 2mm.

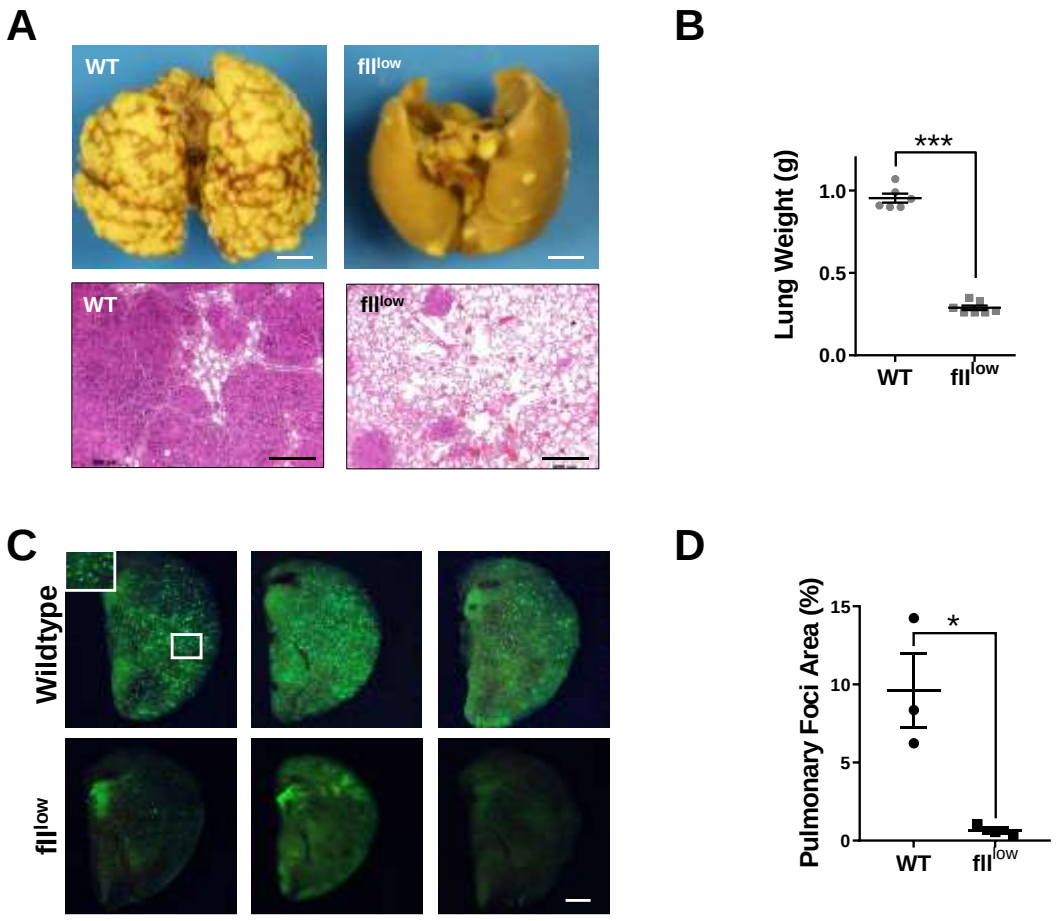

Figure 3.7 Prothrombin promotes mestastatic potential of PDAC cells. (**A, B**) Lung metastasis of KPC2 WT cells injected into C57BL/6 WT mice or *fll*^{low} mice (N=6-7 mice per group). Representative images of lungs harvested 21 days post-injection, total lung weight, and H&E stained lung sections. (**C, D**) Lung metastasis of GFP-expressing KPC2 cells tested in WT and *fll*^{low} mice. Cells were injected into the tail vein and then lungs harvested 24 hr post-injection to establish the degree of cell seeding in the lungs. Scar bar: 2 mm for the lung pictures, 0.5 mm for the H&E staining. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. These experiments were performed in collaboration with Dr. Flick.

3.3 Discussion

TF has long been proposed to influence tumor biology. For example, TF can mediate cell signaling events through TF-Factor VIIa complex formation and subsequent activation of PAR-2 (X. et al., 2004). This receptor complex has been linked to numerous signaling pathways that drive tumor progression, including ERK1/2, JNK, PI3K, and PKCα (Bluff et al., 2008; Hu et al., 2013; Khorana et al., 2007; Rak et al., 2006; Schaffner & Ruf, 2009; H H Versteeg et al., 2000). TF can also exert cell effects by engaging β1 integrins where modulation of integrin binding and integrin-driven signaling is controlled in part by phosphorylation of the short TF-cytoplasmic tail (Belting et al., 2004). The majority of the signaling pathways that are modulated by TF affect cell proliferation *in vitro* (C. Xu et al., 2011; Y.-J. Yu et al., 2014). However in my study, knockdown of TF in mouse PDAC KPC cells did not affect cell proliferation *in vitro*, indicating that mechanisms other than TF-mediated intrinsic signaling drive TF-mediated PDAC tumor growth and metastasis **(Figure 3.3B)**. Beyond cell surface-associated TF, an alternatively spliced soluble form of TF (termed asTF) has also been shown to be secreted by cancer cells, circulating in patient's blood and playing a critical role in pancreatic cancer. Elevated asTF expression in PDAC tumor cells correlates with advanced histological grade and poor patient prognosis (J. et al., 2010; Unruh et al., 2015; van den Berg et al., 2012; Wu et al., 2017) and in pancreatic cancer models asTF can drive inflammatory cell infiltration and increased metastatic potential, further suggesting that TF-mediated signaling may affect the TME component of PDAC tumors and not directly influence PDAC cells (Signaevsky et al., 2008; Unruh et al., 2014). Thus, we hypothesized that TF partners with prothrombin and creates a thrombin enriched TME during PDAC progression and it is this pathway that plays a prominent role in PDAC disease.

The TF downstream factor, Thrombin, has also been linked to cancer progression, however the precise mechanisms by which thrombin activation contributes to lethal pancreatic cancer disease have not been elucidated. Here, we show that genetic or pharmacological reduction of prothrombin suppresses the growth of KPC PDAC tumor cells *in vivo*. More importantly, inhibition of thrombin after implantation of the PDAC cells in the mouse pancreas suppressed tumor growth despite the presence of a complex pancreas TME. These results support the concept of a therapeutic application for PDAC by targeting thrombin. In addition, blocking thrombin in the mouse body also abolished PDAC tumor metastasis to the lung via either inhibition of tumor adhesion or survival of the tumor cells.

Taken together, our study implicating the TF-thrombin axis in PDAC tumor progression adds yet another layer of complexity to the role of TF and thrombin within the TME and tumor cells. Despite these diverse pathways, the different proposed mechanisms are not mutually exclusive and may function in concert. Additional studies will be required to determine if pathways are additive or synergistic and whether any are uniquely functional at distinct stages of PDAC disease. Regardless of the data indicating that the TF-thrombin axis may promote cancer progression, the identification of specific mechanisms and thrombin targets (*e.g.*, fibrinogen, protease-activated receptor [PAR]-1, -3, -4) have largely remained open questions.

CHAPTER 4. PAR-1 SIGNALING AS A GATEWAY TO PANCREATIC CANCER GROWTH AND METASTASIS

4.1 Introduction

Our studies have shown that the TF-thrombin axis is critical for PDAC tumor progression and that thrombin has multiple downstream targets including fibrinogen in the TME and protease activated receptors (PARs) on the cell surface. Based on a number of genetic studies we hypothesize that thrombin primarily promotes PDAC progression through activation of PARs (**Figure 4.1**).

Protease activated receptors (PARs) belong to the G-protein coupled receptor super family which contain N-termini domains with proteolytic cleavage sites, a seven transmembrane domain and a C-terminus signaling domain. Once thrombin or other proteases cleave the N-terminus of PARs on the cell surface, the remaining portion of the N-terminus protein serves as a "tether" ligand that binds back towards the transmembrane domain to initiate downstream G protein signaling events. Within minutes after the proteolytic cleavage, PARs internalize through endocytosis and traffic to lysosomes for degradation. Thus, constant PAR signaling relies solely on newly synthesized PARs and presence of thrombin. To insure a steady supply of PARs on the cell surface thrombin stimulation exerts a positive feedback loop downstream of PARs. For instance, thrombin activates PAR-1 signaling which induces PAR-1 gene expression through the Ras/Raf/MAP kinase pathway (Ellis et al., 1999). Independent from thrombin, PDAC cancer cells have abnormal activation of Ras downstream signaling (*e.g.*, oncogenic KRas), which potentially serves as a source of persistent PAR-1 induction. Taken together, oncogenic KRas which is a hallmark of PDAC cells, likely utilizes the TF-Thrombin-PAR-1 positive feedback loop to drive

robust KRas activity, PAR-1 expression, constant TF-thrombin-PAR-1 signaling and ultimately promote PDAC cell tumor growth and metastasis (**Figure 4.1**).

In order to test these possibilities, I used shRNA knockdown and CRISPR knockout strategies to target PAR-1 in mouse KPC cells to evaluate how downregulating PAR-1 on tumor cells affects tumor growth and metastasis. I also utilized a tetracycline inducible system to rescue PAR-1 expression in Par-1^{KO} cells to determine the dependency of PAR-1 activity for tumor growth. In addition, I turned on and off PAR-1 in KPC cells during tumor progression to test the therapeutic potential of targeting PAR-1 and characterized the TME of PDAC tumors with or without PAR-1. My studies support a model where PAR-1 activity plays a central role in PDAC disease.

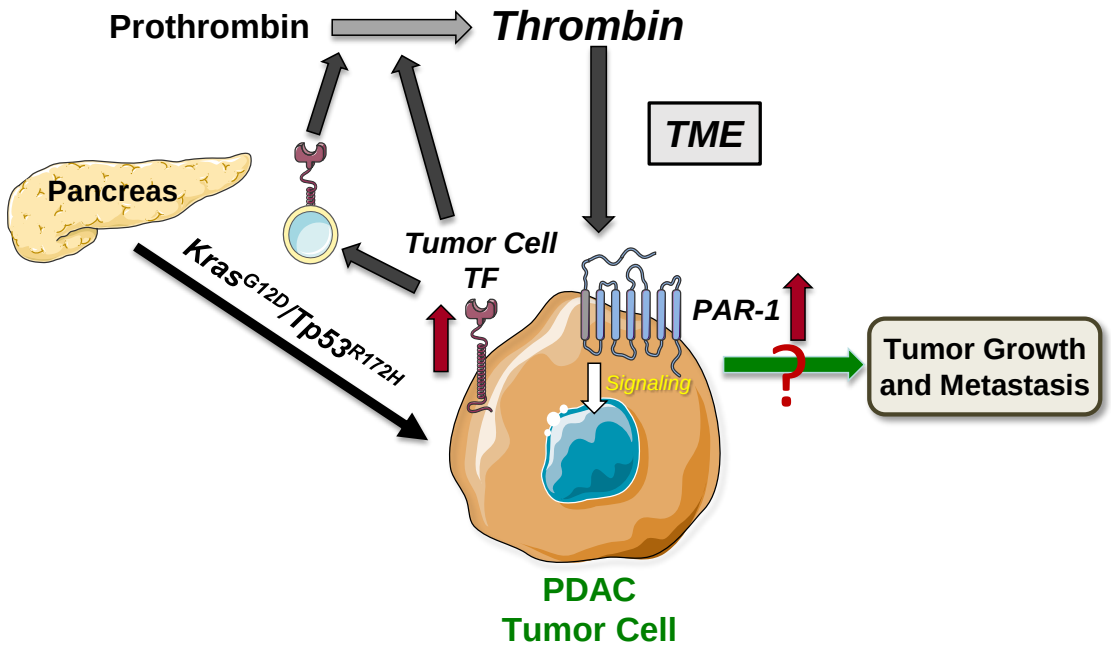

Figure 4.1 Model of the current working hypothesis. The TF-Thrombin axis promotes PDAC tumor growth and metastasis through the PAR-1 receptor on the tumor cell surface in an autocrine manner.

4.2 Results

4.2.1 *Cancer cell derived PAR-1 drives PDAC tumor growth and metastasis*

There are a total of 4 PARs in the protease activated receptor family, PAR-1,-2,-3 and -4. Thrombin is able to activate PAR-1, -3 and -4 but not PAR-2 (Soh et al., 2010). In order to determine which PARs would be the primary target for thrombin activation in PDAC, transcript levels of the Par gene family were quantified by RT-qPCR. As expected, WT pancreas tissue exhibited nearly undetectable expression of all Par family members (**Figure 4.2A**). In contrast, primary mouse PDAC tumor tissue and individual PDAC tumor cell lines expressed high levels of *F2r* (PAR-1 gene). Gene transcripts for PAR-2, -3, and -4 (*F2rl1*, *F2rl2* and *F2rl3*) were also elevated in primary PDAC tumors and KPC tumor cell lines but at significantly lower levels than observed for PAR-1 when compared to normal pancreata expression (**Figure 4.2A**). Indeed, elevated transcript levels of the other thrombin sensitive PARs (*F2rl2, F2rl3*) were substantially lower than those for *F2r*, suggesting that PAR-1 is likely the primary tumor cell target of thrombin.

In addition to the mouse PDAC lines, I also screened for *F2R* expression in four of the most common human PDAC cell lines (BXPC3, Panc1, AsPC1, and Miapaca2). As shown in **Figure 4.2B**, the highly aggressive, poorly differentiated Panc1, AsPC1 and Miapaca2 lines exhibited significantly elevated levels of *F2R* expression when compared to the immortalized normal pancreas ductal cell line HPDE6. In contrast, the weakly tumorigenic, moderately differentiated BXPC3 line exhibited decreased PAR-1 expression. Interestingly, Panc1, AsPC1 and Miapaca2 contain activated KRas oncogenes whereas BXPC3 retains wildtype KRas alleles, consistent with the findings that the coagulation cascade factor PAR-1 is downstream of oncogenic KRAS. Finally, to confirm PAR-1 protein expression in human PDAC and in mouse PDAC models, immunohistochemistry was performed on patient samples and on mouse PDAC samples.

As shown in **Figure 4.2C, D**, robust PAR-expression was observed in PDAC patient samples and in mouse PanIN and PDAC epithelial lesions.

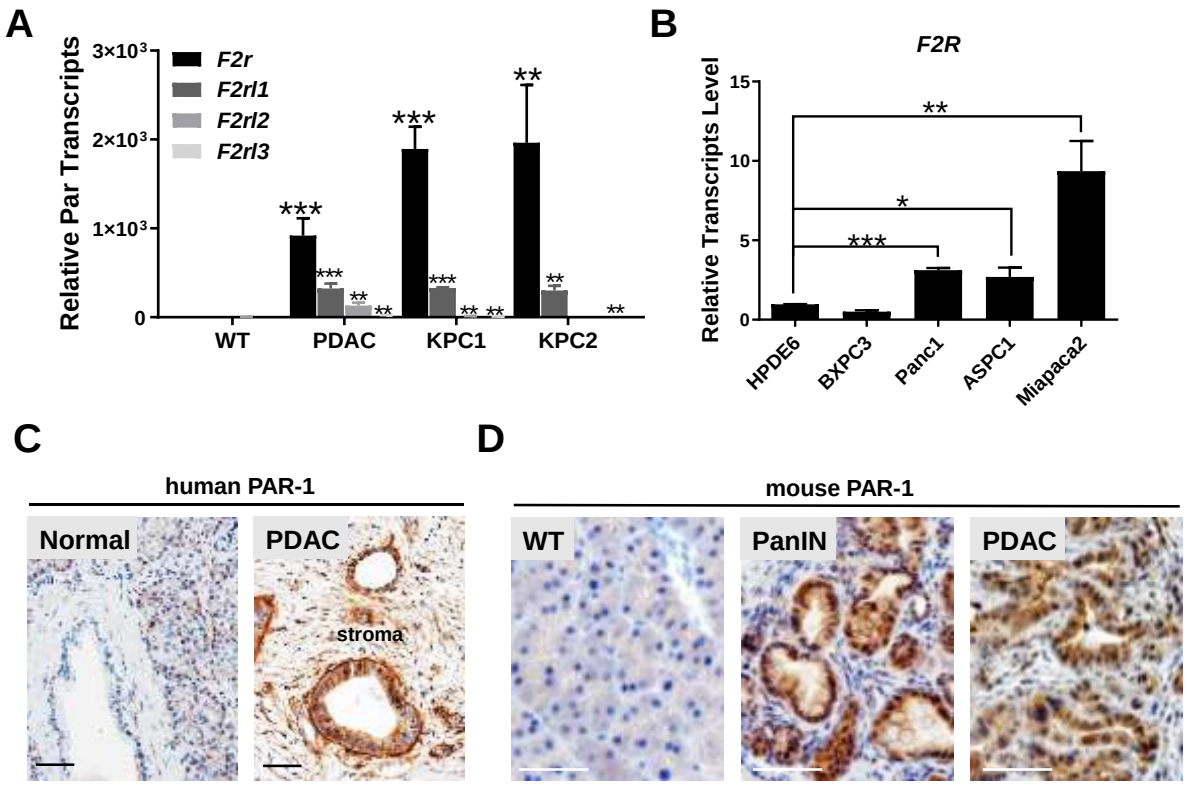

Figure 4.2 PAR-1 is highly expressed in transformed PDAC epithelial cells. (A) RT-qPCR analysis of transcripts for *F2r*, *F2rl1*, *F2rl2* and *F2rl3* in mouse WT pancreas, PDAC, KPC1 and KPC2 samples (N=3 per group). Statistical significance was calculated comparing relative transcript levels of each gene in WT pancreata vs. PDAC, KPC1 and KPC2 groups. **(B)** RT-qPCR analysis of transcripts of human *F2R* in HPDE6, BXPC3, Panc1, ASPC1 and Miapaca2 cell lines. (**C&D**) PAR-1 immunohistochemistry in normal human pancreas or PDAC patient samples and in mouse WT pancreas, PanIN lesions, and PDAC. Scale bars: 50 µm. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

After establishing the PAR-1 expression profile, we turned to determine if tumor cell derived PAR-1 is a critical receptor for influencing tumor growth. Stable shPar-1 knockdown KPC2 lines were generated using lentiviral transduction. As shown in **Figure 4.3A,** Par-1 expression in KPC2 shPar-1 lines was successfully knocked down to only 15-20% when compared to the shControl line. I next examined the cell proliferation rate of these cell lines. Interestingly, proliferation indices and doubling time of KPC2 shControl and KPC2 shPar-1 cells grown *in vitro* were similar, suggesting that Par-1 alone does not regulate aspects of the cell cycle (**Figure 4.3B, 4.3C**).

To determine whether reduction of Par-1 in KPC2 cells can affect tumor growth and metastasis in mice, subcutaneous allograft assays and lung metastasis assays were performed in C57Bl/6 mice in collaboration with Dr. Matthew Flick. The shPar-1 cells displayed reduced tumor growth when compared to growth rates obtained testing KPC2 shControl cells (**Figure 4.4A**), resulting in a significantly lower final tumor mass for the shPar-1 cells (**Figure 4.4B**). Given that the cell proliferation indices were similar between KPC2 shControl and shPar-1 cells, PAR-1 mediated PDAC tumor growth might result from interaction with the TME other than simply to modulate cell proliferation. In support of this hypothesis, we found that KPC2 shPar-1 cells exhibited a significantly reduced metastatic potential where there were less tumor nodules in the lung as evidenced by the uninterrupted pulmonary area (**Figure 4.4C**) and subsequently reduced

lung weight (**Figure 4.4D**). Collectively, these results demonstrate that cancer cell- PAR-1 promotes PDAC tumor growth and hematogenous metastatic potential.

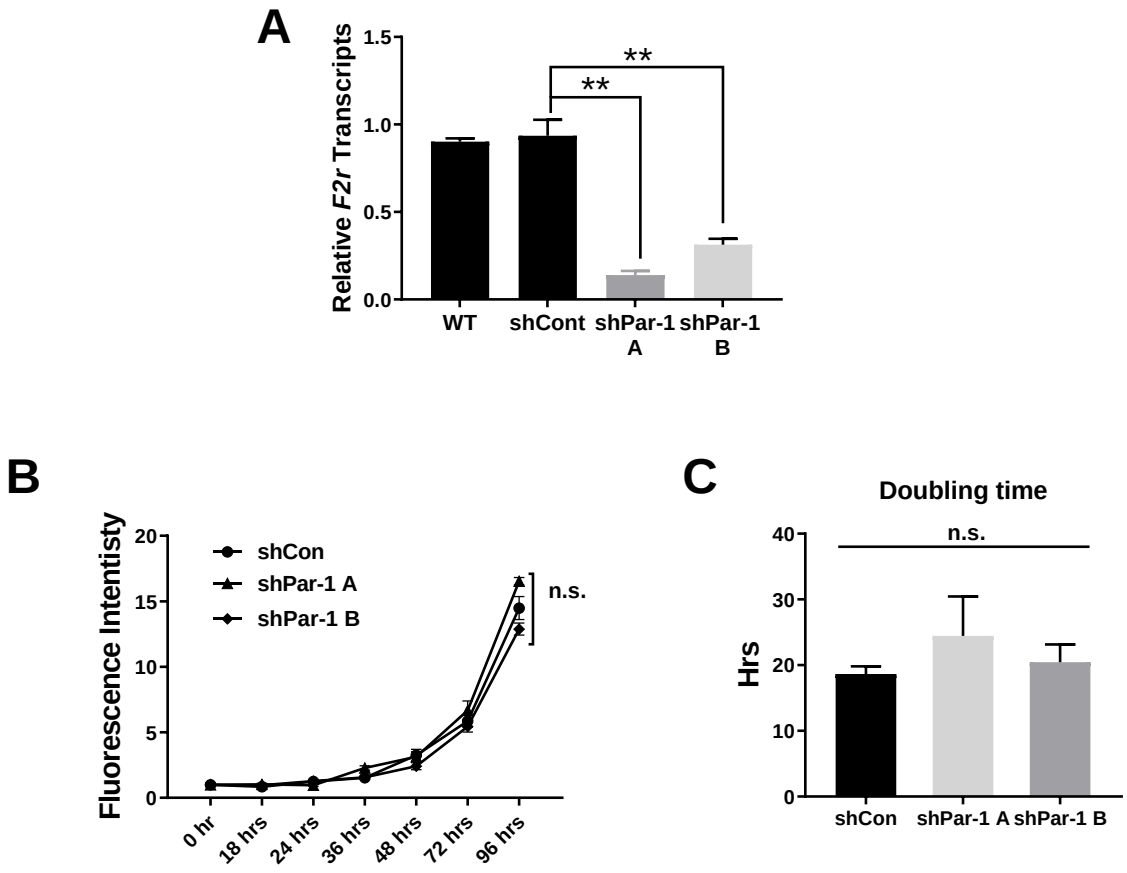

Figure 4.3 Reduction of PAR-1 expression did not alter cell proliferation *in vitro*. (A) RT-qPCR analysis for *F2r* transcripts in KPC2 WT, KPC2 shControl and KPC2 shPar-1 'knock down' cell lines (N=3). shPar-1 A and shPar-1 B represent two different shRNA sequences. (**B, C**) Growth curve and doubling times for KPC2 WT, KPC2 shCont and shPar-1 cell lines in culture. ** $P < 0.01$; n.s.: not significant.

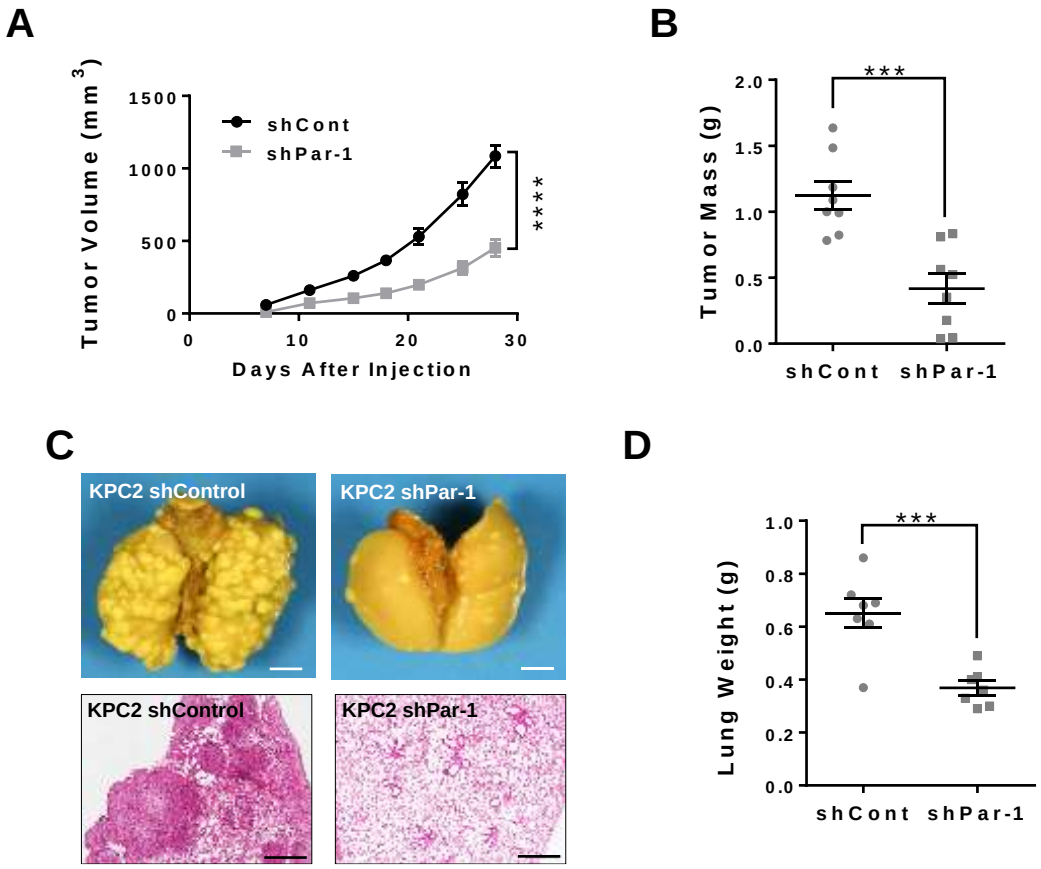

Figure 4.4 Reduction of tumor cell PAR-1 expression diminishes PDAC tumor growth and metastasis. (A, B) Subcutaneous allograft assays using KPC2 shCont and KPC2 shPar-1 lines in C57BL/6 mice. Tumor sizes were measured over time and tumor mass quantified at the day of sacrifice (N=8 mice per group). **(C, D)** Lung metastasis of KPC2 shControl and KPC2 shPar-1 cells in C57Bl/6 mice (N=7 per group). Scale bar: 2 mm for the whole mount lung pictures, 0.5 mm for the H&E sections. *** $P < 0.001$; **** $P < 0.0001$; These studies were performed in collaboration with Dr. Flick at CCHMC.

4.2.2 *PDAC tumor growth is dependent on cancer cell derived PAR-1 expression*

In order to determine the role of PAR-1 in tumor growth in a clean genetic setting I generated KPC2 PAR-1 edited cell lines (Par-1^{KO1}, Par-1^{KO8}) using a derivative of CRISPR-Cas9 to introduce a deletion in the 5' portion of exon 1, resulting in loss of the initiator methionine codon and signal peptide sequence (**Figure 4.5A**). RT-qPCR analysis revealed that these deletions resulted in substantial transcript instability when compared to the wildtype allele (**Figure 4.5B**).

To confirm the absence of functional PAR-1 protein, KPC2 WT, KPC2 cells transfected with Cas9 cDNA (KPC2 Cas9) and KPC2 Par-1^{KO1} and KPC2 Par-1^{KO8} lines were treated ± thrombin. Thrombin stimulation led to induction of phospho-Erk activation for KPC WT and KPC2 Cas9 control cells, whereas KPC2 Par-1^{KO1} and Par-1^{KO8} cells failed to respond to thrombin, confirming the absence of functional PAR-1 signaling in these cells (**Figure 4.5C**).

We next tested cell proliferation rates *in vitro* and anchorage independent cell growth using soft agar assays. As shown in **Figure 4.6A, B**, KPC2 Par-1^{KO1} and KPC2 Par-1^{KO8} cells exhibited near identical growth rates and doubling times when compared to WT KPC2 and control Cas9 cells, which is consistent with the PAR-1 knockdown phenotype (see **Figure 4.3**). In soft agar assays, KPC2 Par-1^{KO1} and KPC2 Par-1^{KO8} lines also produced similar numbers and sizes of distinct colonies to that of the KPC2 Cas9 cell line, indicating that PAR-1 is dispensable for anchorage-independent growth *in vitro* (**Figure 4.6C, D**). Collectively, inactivating PAR-1 does not affect cell proliferation and anchorage independent cell growth *in vitro*.

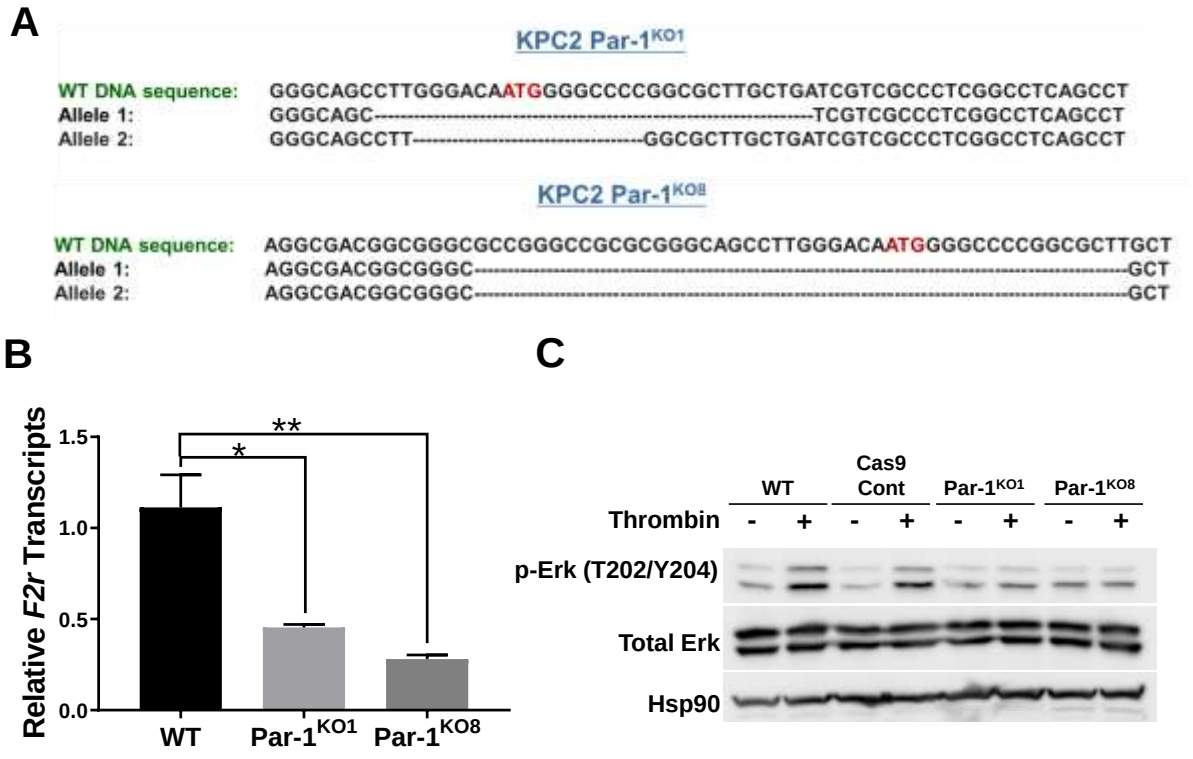

Figure 4.5 PAR-1 expression was impaired and activity was abolished in KPC2 Par-1^{KO1} and KPC2 Par-1^{KO8} cell lines. (A) DNA sequence for KPC2 Par-1^{KO1} and KPC2 Par-1^{KO8} cell lines showing the DNA deleted regions. The initiation ATG start codon is indicated in red in the DNA sequence. **(B)** RT-qPCR of *F2r* transcripts from KPC2 WT, KPC2 Par-1^{KO1}, and KPC2 Par-1^{KO8} cells (N=3 per group). **(C)** Immunoblots for MAPK pathway activation of KPC2 WT, KPC2 Cas9 control, KPC2 Par-1^{KO1}, and Par-1^{KO8} lines ± thrombin. Hsp90 serves as a loading control. * $P < 0.05$; ** $P < 0.01$.

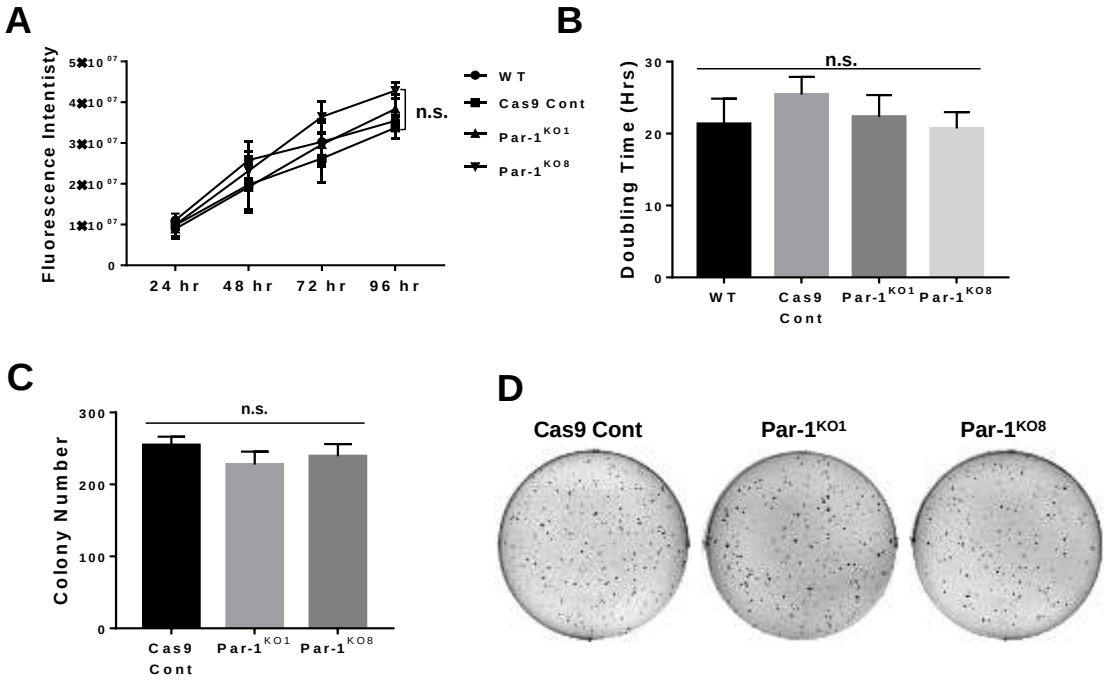

Figure 4.6 Elimination of PAR-1 activity in KPC2 cells does not alter cell proliferation or anchorage-independent growth *in vitro*. (A) Growth curves for KPC2 WT, KPC2 Cas9 control, KPC2 Par-1^{KO1}, and KPC2 Par-1^{KO8} cells (N=3 per group). **(B)** Cell doubling times for the KPC2 WT, KPC2 Cas9 control, KPC2 Par-1^{KO1}, and KPC2 Par-1^{KO8} lines (N=3 per group). **(C)** Soft agar colony formation for the KPC2 Cas9 Control, Par-1^{KO1}, and Par-1^{KO8} lines (N=3 per group). Total colony numbers were counted using ImageJ. **(D)** Representative images of soft agar colony formation for the KPC2 Cas9 Control, Par-1^{KO1}, and Par-1^{KO8} lines. n.s. - not significant.

We next tested the tumor growth potential of these cell lines *in vivo*. In allograft tumor studies, KPC2 Par-1^{KO8} cells exhibited a dramatic reduction in tumor growth rates, which translated to a significant reduction in final tumor mass compared to the Cas9 line **(Figure 4.7A, B)**. Par-1^{KO1} cells exhibited an even more impressive phenotype as these cells produced little to no tumor tissue 4 weeks post-injection. Despite the tumor cell derived PAR-1, stromal and immune cells in the TME also express PAR-1 and have been shown to promote PDAC progression (Queiroz et al., 2014). To determine the role of stromal PAR-1 in PDAC tumor progression, we partnered with Dr. Flick and tested the stroma PAR-1 activity by implanting WT KPC2 cells into C57Bl/6 WT vs. *F2r*$^{-/-}$ mice. Surprisingly, no difference in tumor growth was observed with KPC2 cells in *F2r*$^{-/-}$ mice when compared to the same cells in wiltype C57Bl/6 mice (**Figure 4.7C, D**). These results support the concept that KPC tumor cell PAR-1 signaling is a potent determinant of PDAC tumor growth *in vivo* despite robust proliferative activity *in vitro*.

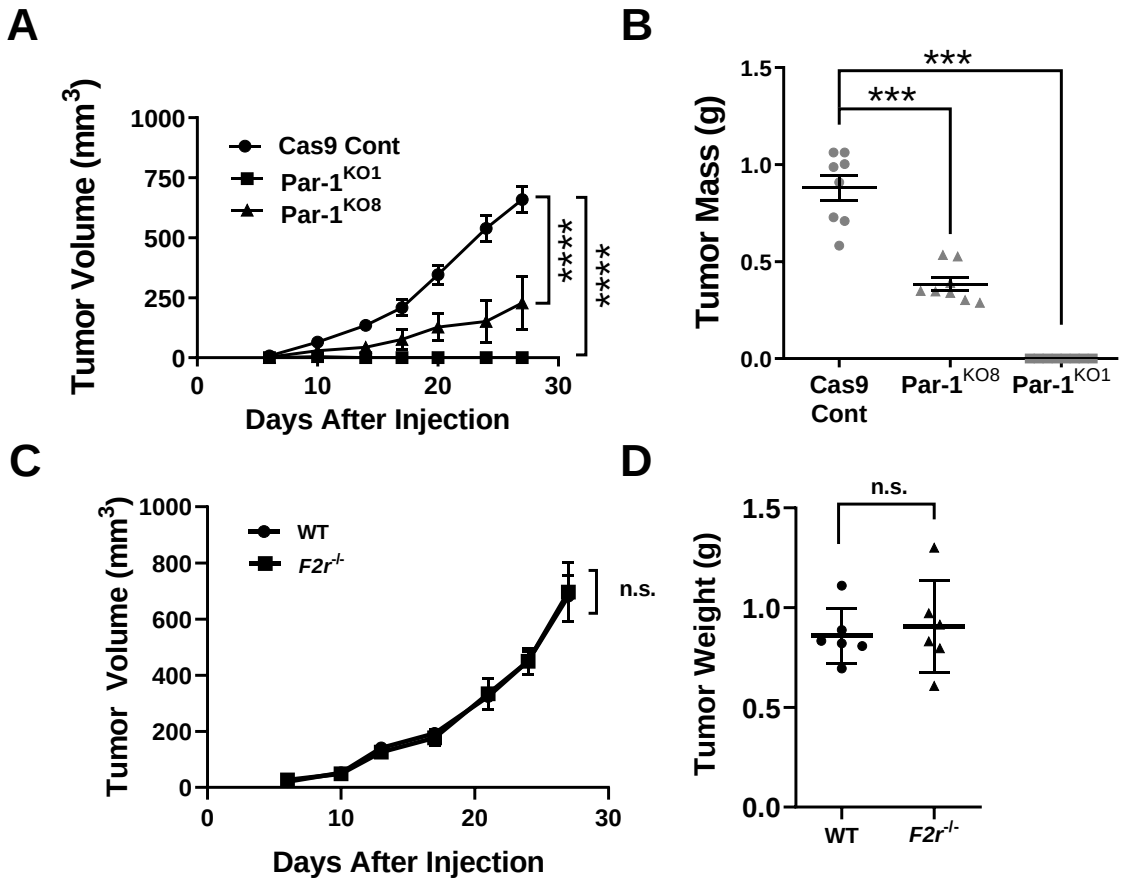

Figure 4.7 Elimination of KPC2 tumor cell PAR-1 activity, not stroma PAR-1 activity, significantly reduces tumor growth *in vivo*. (A, B) Analysis of tumor growth and final tumor mass following subcutaneous injection of KPC2 Cas9 Control, KPC2 Par-1^{KO1}, and KPC2 Par-1^{KO8} lines into C57Bl/6 mice (N=8 mice per group). (**C, D**) Analysis of tumor volume and final tumor mass following subcutaneous injection of KPC2 shControl cells into C57Bl/6 WT and *F2r*$^{-/-}$ mice. *** $P < 0.001$; **** $P < 0.0001$; n.s. - not significant.

To confirm that the failure of tumor growth potential in KPC Par-1^{KO} cells was indeed due to loss of PAR-1 activity and not to an off-target effect, we introduced a doxycycline (Dox) inducible Par-1^{Myc} transgene (Tg) into KPC2 Par-1^{KO1} cells (resulting in the derivative line KPC2 Par-$1^{KO/Tg}$). As expected, PAR-1^{Myc} protein was not detected in KPC WT cells or in the KPC2 Par-$1^{KO/Tg}$ line maintained in the absence of Dox (**Figure 4.8A**). However, treatment of KPC2 Par-$1^{KO/Tg}$ cells with Dox led to rapid induction of PAR-1^{Myc} that localized predominantly to the cell surface (**Figure 4.8A**). Transcriptionally, KPC2 Par-$1^{KO/Tg}$ cells expressed Par-1^{Myc} transcripts at levels comparable to that observed for the endogenous *F2r* gene in KPC2 WT cells (**Figure 4.8B**). We next determined the functionality of the PAR-1^{Myc} protein by treating KPC2 Par-$1^{KO/Tg}$ cells with thrombin following Dox induction. Under these conditions, thrombin addition led to the expected PAR-1^{Myc}-dependent activation of phospho-Erk (**Figure 4.8C**). No increase in phospho-Erk was observed for KPC2 Par-$1^{KO/Tg}$ cells treated with thrombin in the absence of simultaneous Dox treatment, confirming that PAR-1 protein is required to elicit a thrombin response in these cells.

The potential of KPC2 Par-$1^{KO/Tg}$ cells to form tumors in mice following Dox-induced PAR-1^{Myc} was next evaluated. Studies have shown that Dox can directly inhibit PAR-1 by binding to the transmembrane domain and block the tether ligand, which results in inhibition of tumor growth *in vivo* (Zhong, Chen, Qin, et al., 2017; Zhong, Chen, Zhang, et al., 2017). Thus, we first evaluated whether Dox treatment using Dox chow can affect tumor growth in our setting. As shown in **Figure 4.9A, 4.9B**, Dox did not alter the normal pattern of tumor progression for PDAC cells, where WT KPC2 cells continued to form large tumors whereas KPC2 Par-1^{KO1} cells generated only small

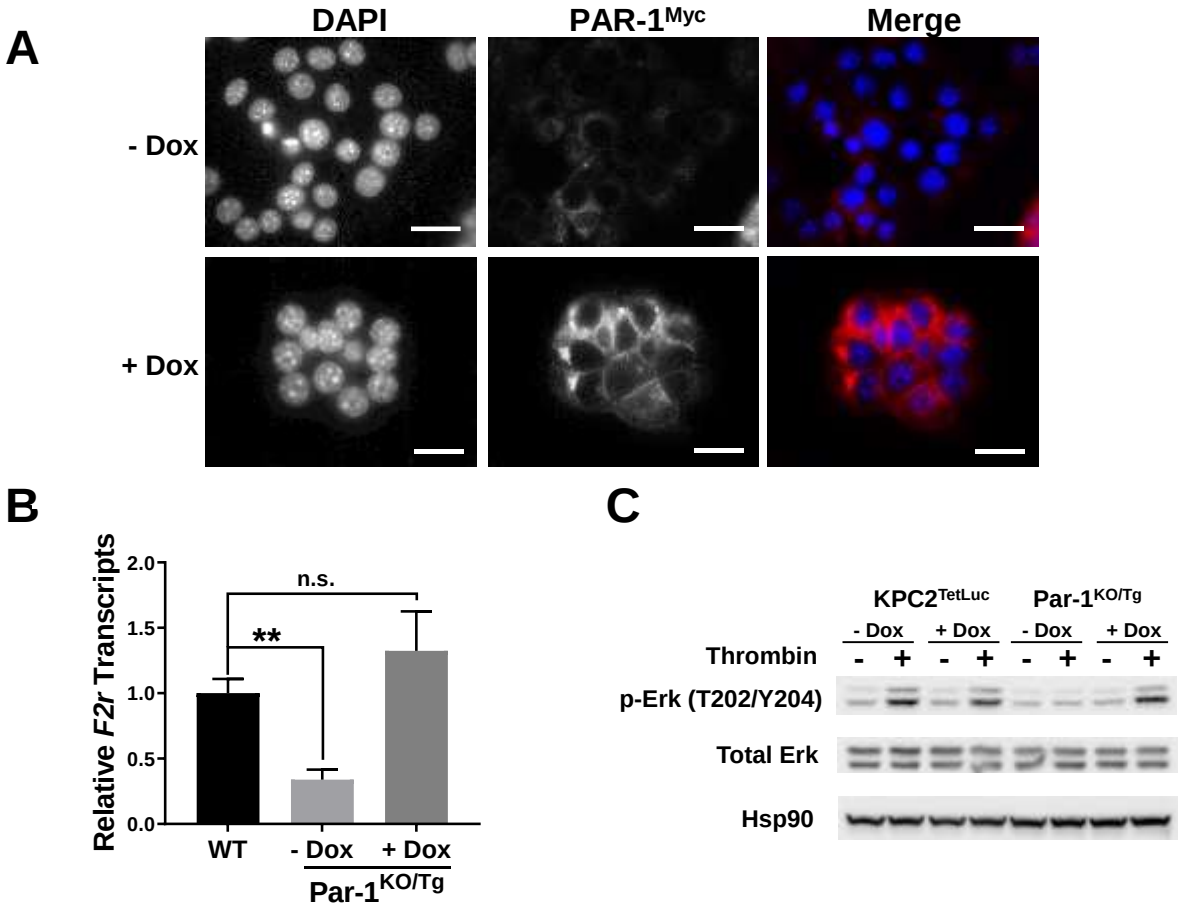

Figure 4.8 Ectopic PAR-1 activity restores KPC2 Par-1^{KO} cell responses to thrombin. (A) Anti-Myc immunofluorescence of KPC2 Par-1$^{KO/Tg}$ cells treated ± Dox. **(B)** RT-qPCR for endogenous and Tg *F2r* transcripts comparing KPC2 WT and KPC2 Par-1$^{KO/Tg}$ cells ± Dox stimulation (N=3 per group). **(C)** Immunoblot for phospho-ERK in KPC2 cells containing a Tet-inducible luciferase expression vector (KPC2^{TetLuc}) and KPC2 Par-1$^{KO/Tg}$ cells stimulated ± Dox and ± thrombin. ** $P < 0.01$; n.s. - not significant.

tumor nodules, regardless of Dox status during the time period we tested. Thus, Dox treatment in our setting did not affect the tumor growth properties of PDAC cells.

We next tested the KPC2 Par-$1^{KO/Tg}$ cells in comparative tumor growth studies with WT mice ± Dox. As presented in **Figure 4.9C, 4.9D**, KPC2 Par-$1^{KO/Tg}$ cells failed to produce significant tumors when mice were maintained in the absence of Dox. However, when the same KPC2 Par-$1^{KO/Tg}$ cells were tested in mice maintained on Dox chow, large tumors developed. Importantly, the growth rate of the KPC2 Par-$1^{KO/Tg}$ cells in mice with Dox was similar to that observed with KPC2 WT cells. These results show that the limited tumor growth of KPC2 Par-1^{KO1} cells is fully restored by the re-introduction of PAR-1, emphasizing that PDAC tumor growth is dependent on cancer cell derived PAR-1 expression.

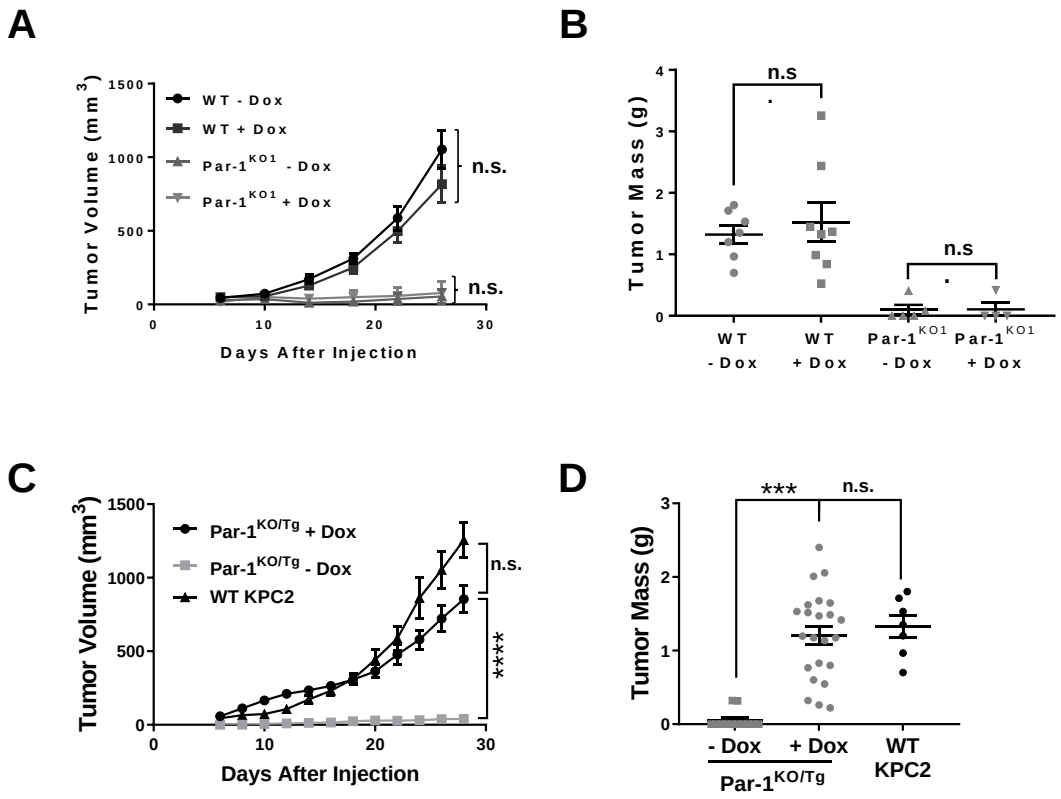

Figure 4.9 Ectopic PAR-1 activity restores KPC2 Par-1^{KO} cell tumor growth. (**A, B**) Analysis of tumor volume and final tumor mass following subcutaneous injection of KPC2 WT cells or KPC2 Par-1^{KO1} cells ± Dox (N=8 mice per group). **(C, D)** Analysis of tumor volume and final tumor mass following subcutaneous injection of KPC2 Par-1$^{KO/Tg}$ cells into cohorts of mice treated ± Dox chow (N=12 mice per group) and WT KPC2 cells (N=6 mice). *** P<0.001; **** P<0.0001; n.s. - not significant.

To evaluate the possibility of targeting PAR-1 as a therapeutic in the clinic, we needed to determine how tumors react when PAR-1 is removed in an established tumor. Therefore, I repeated the allograft experiment and switched the Dox food to normal chow in KPC2 Par-$1^{KO/Tg}$ cells to turn off PAR-1 expression at 11 days, when the tumor had grown to a visible size (+Dox vs. +Dox/-Dox). As a complementary experiment, I switched the normal chow to Dox food in KPC2 Par-$1^{KO/Tg}$ cells at 11 days to investigate whether KPC2 Par-$1^{KO/Tg}$ cells in the absence of Dox survive at the injection site and whether they could then reform a tumor after turning on PAR-1 expression (-Dox vs. -Dox/+Dox). As shown in **Figure 4.10A, 4.10B**, in the +Dox vs. +Dox/-Dox groups, no statistical differences were observed in tumor volume and mass between +Dox and +Dox/-Dox groups, suggesting that turning off PAR-1 expression in KPC2 Par-$1^{KO/Tg}$ cells in established tumors fails to inhibit further tumor growth *in vivo.* However, the tumor volume and mass variation is higher in the +Dox/-Dox group than in the -Dox group. After close examination, I divided the data in the +Dox/-Dox group into three subgroups based on three tumor growth properties: (i) decline in tumor growth, (ii) continue to growth, and (iii) stagnant tumor growth. As presented in **Figure 4.10C, 4.10D**, tumors that were approximately less than 250 mm^3 remained at the same size or regressed over time upon removal of PAR-1 expression. However, tumors larger than 250 mm^3 continued to grow, regardless of turning off PAR-1 expression. These results suggest that PAR-1 is critical for small tumor growth *in vivo* where the establishment of the TME may be distinct from the TME with larger tumor size. In the -Dox vs. -Dox/+Dox groups, all mice in -Dox cohort exhibited no tumor formation over the 28 days. Even though there was no statistical significance observed in tumor growth when compared to

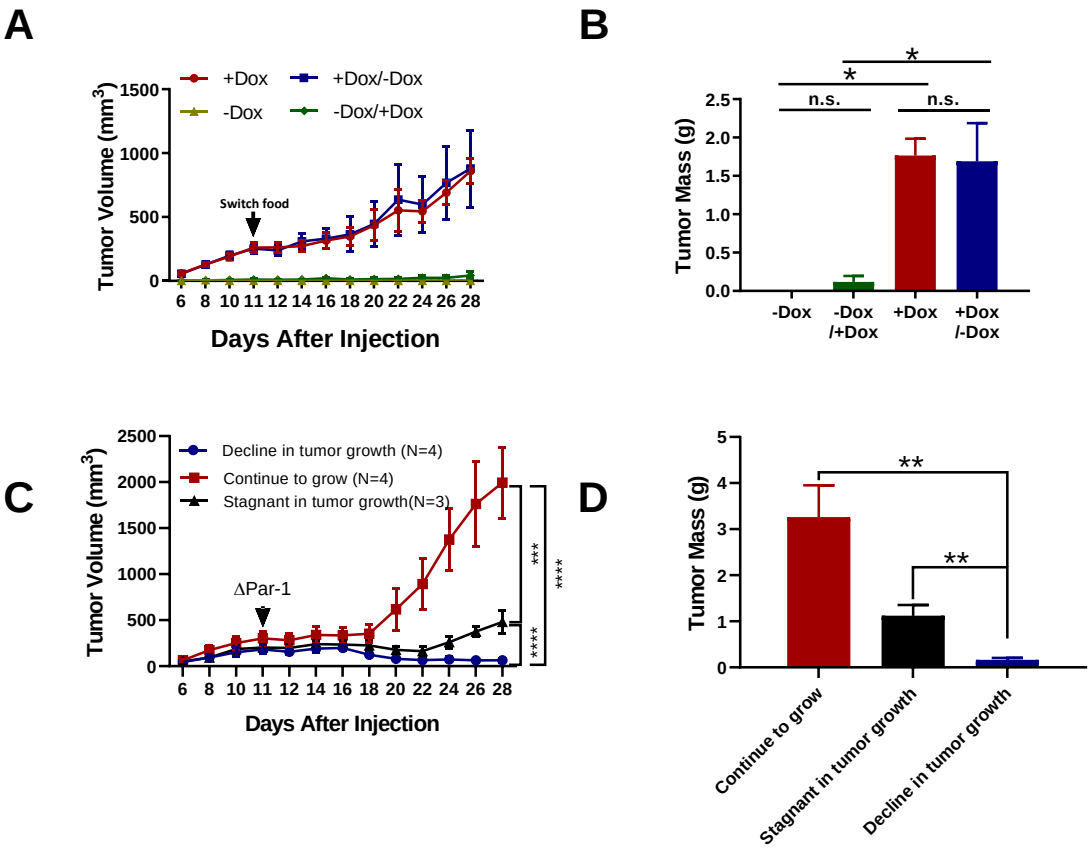

Figure 4.10 Removal of tumor cell PAR-1 activity in an established tumor alters tumor growth behavior in a tumor size dependent manner. (A, B) Analysis of tumor volume and final tumor mass following subcutaneous injection of KPC2 Par-1^{KO1} cells ± Dox with the option of switching food at 11 days (N=8-12 mice per group). **(C, D)** Tumor volume and final tumor mass of the +Dox/-Dox group were categorized into three groups based on tumor growth properties. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; n.s. - not significant.

the -Dox/+Dox group, 2 out of 6 mice in the -Dox/+Dox group showed small tumor grow (100 and 150 mm^3), indicating that some of the KPC2 Par-$1^{KO/Tg}$ cells managed to survive and stay non-proliferative during the first 11 days and these cells then were able to enter a proliferative phase upon PAR-1 induction (**Figure 4.10A, 4.10B**).

Pancreas TME is critical for PDAC aggressiveness and it also is responsible for inefficient drug delivery (Jacobetz et al., 2013). Therefore, in order to test PAR-1 dependent tumor growth in amore clinically relevant setting, we next performed orthotopic injections of KPC2 WT cells or KPC2 Par-$1^{KO/Tg}$ cells into the mouse pancreas. Animals receiving KPC2 Par-$1^{KO/Tg}$ cells were fed ± Dox to evaluate the impact of tumor cell PAR-1 expression on tumor growth within the pancreas microenvironment. The orthotopic model perfectly mimicked results from the subcutaneous tumor model where mice injected with KPC2 Par-$1^{KO/Tg}$ cells in the absence of Dox treatment exhibited minimal tumor formation (**Figure 4.11A, B**). In contrast, a robust tumor growth response was obtained in Dox-treated mice tested with KPC2 Par-$1^{KO/Tg}$ cells. Again, tumor growth was equivalent to that observed following orthotopic injection of KPC2 WT cells (**Figure 4.11A, B**).

We next evaluated the contribution of tumor cell PAR-1 expression to PDAC-induced mortality. KPC2 WT cells and KPC2 Par-1^{KO1} cells were introduced into the pancreata of adult C57Bl/6 mice and mouse survival was followed over time. As presented in **Figure 4.11 C**, mice injected with KPC2 WT cells uniformly succumbed to the PDAC challenge with a median survival of 37 days. In contrast, mice tested with KPC2 Par-1^{KO1} cells displayed a significantly prolonged lifespan with a median survival of 69 days. Indeed, 2 of 12 mice injected with KPC2 Par-1^{KO1} cells had no tumors and survived through the entire evaluation period of 106 days. Collectively, these

findings confirm that reduced tumor growth displayed by PDAC cells in which PAR-1 signaling has been eliminated leads to significantly improved survival for tumor-bearing hosts.

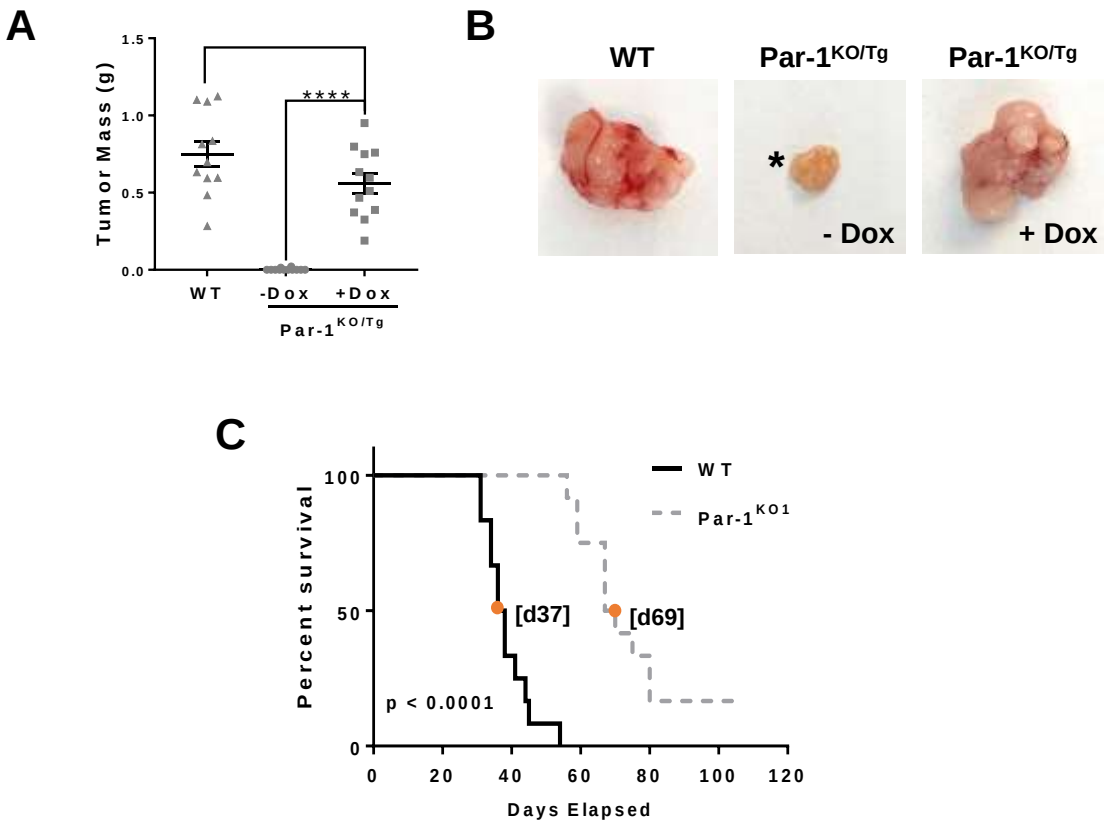

Figure 4.11 Expression of PAR-1 drives tumor growth in the pancreas microenvironment, resulting in poor host survival. (A) KPC2 WT or KPC2 Par-$1^{KO/Tg}$ cells were injected into the pancreas and mice were treated ± Dox (N=12 mice per group). Pancreas tumors were harvested at 21 days post-surgery. **** P<0.0001; n.s. - not significant. **(B)** Representative examples of isolated tumors from the pancreas of mice 21 days post-surgery. Asterisk indicates one of only two tumors generated out of a cohort of 12 mice from the Par-$1^{KO/Tg}$ cells in the absence of Dox. The remaining 10 animals failed to develop visible tumors within the 21 day period. Scale bar: 2 mm. **(C)** Survival study following orthotopic pancreas injection of KPC2 WT vs. KPC2 Par-1^{KO1} cells (N=12 mice per group). Median survival times are indicated for each study (red dot).

4.2.3 *Tumor cell PAR-1 signaling supports tumor growth by influencing the immune system in the TME*

To begin to determine the mechanism by which tumor cell thrombin-PAR-1 signaling promotes tumor growth, we first investigated *in vivo* changes in cell proliferation and apoptosis. Despite significant changes in overall tumor growth, no genotype-dependent differences were detected in the amount of immunohistochemical staining for the proliferation marker phospho-histone 3 (pH3) in sections of KPC2 WT and KPC2-Par-1^{KO1} or KPC2-Par-1^{KO8} tumors grown in WT mice for 1 week following subcutaneous injection (**Figure 4.12A**). Similar findings were observed for KPC2 WT and KPC2-shPar-1 following subcutaneous injection using Ki67 as a marker (Figure **4.12B)**. A significant difference in staining for the apoptosis marker cleaved caspase-3 (CC3) was observed in that a higher fraction of cells were CC3+ in tumor sections of subcutaneously grown KPC2-Par-1^{KO1} or KPC2-Par-1^{KO8} compared to control KPC2-Cas9 cells (**Figure 4.12C**). The increase in caspase activity in cells lacking PAR-1 suggests that the different tumor growth observed in PAR-1 knockout cells is mainly due to cancer cell apoptosis *in vivo*.

To begin to determine if there are potential differences in the tumor microenvironment, a series of immunohistochemical analyses were performed on KPC2 and KPC2-shPar-1 tumors grown for 2 weeks following subcutaneous injection. Whereas tumors grown from KPC2 WT cells had significant numbers of KPC2 cells as shown by cytokeratin 19 (K19) staining, tumors grown from KPC2-shPar-1 cells had very few K19+ KPC2-shPar-1 cells within the tumor area (**Figure 4.13**). However, one key difference was the presence of immune cells. In KPC2 WT tumors, activated macrophages (Iba-1+ cells) and T cells (CD3+ cells) were generally restricted to the perimeter of the tumor mass whereas in KPC2-shPAR-1 tumors activated macrophages and T cells were observed throughout the tumor tissue (**Figure 4.13**). Collectively, these studies suggest a

possible mechanism whereby loss or reduction of PAR-1 in the tumor cells results in a failure of tumor growth coupled to enhanced immune-mediated clearance of tumor cells.

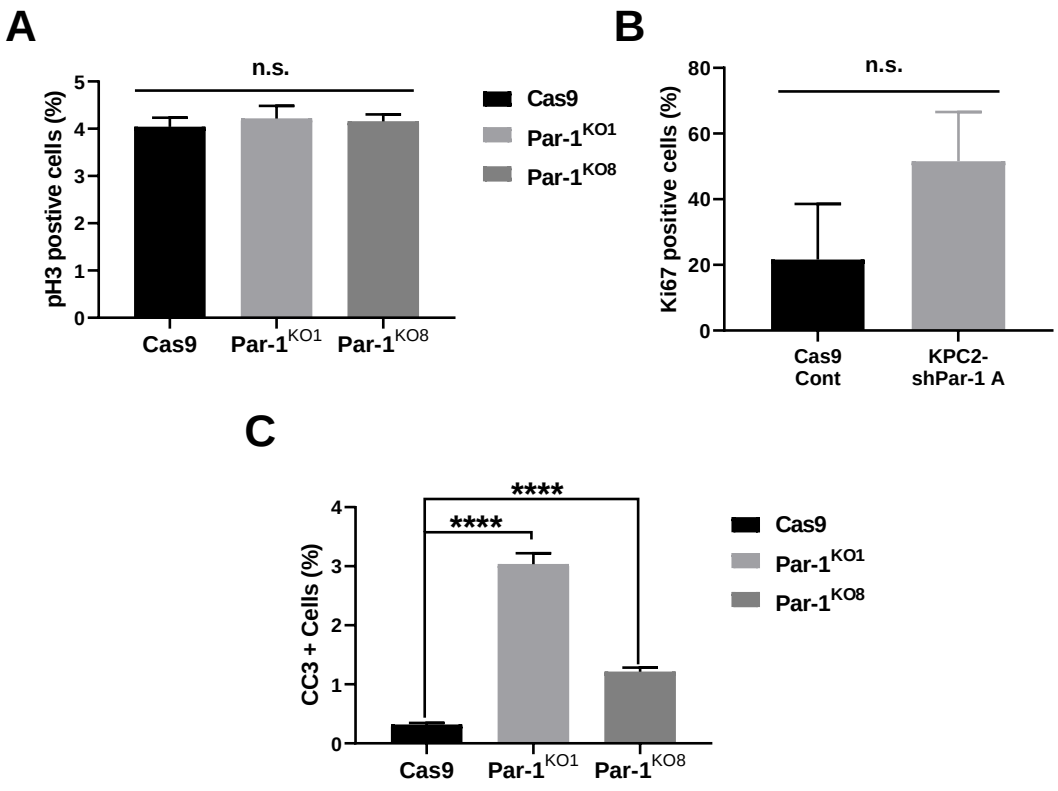

Figure 4.12 Reduction of Par-1 in KPC cells leads to induced apoptosis *in vivo*. (A) pH3 staining analysis of tissue sections of KPC2-Cas9, KPC2-Par-1^{KO1}, and KPC2-Par-1^{KO8} (n=3-4 mice/group) tumor tissue 1 week following subcutaneous injection into WT C57Bl/6 mice. **(B)** Ki67 staining analysis of tissue sections of KPC2-shCon and KPC2-shPar-1 (n=4 mice/group) tumor tissue 2 weeks following subcutaneous injection. **(C)** Cleaved caspase-3 (CC3) staining analysis of tissue sections of KPC2-Cas9, KPC2-Par-1^{KO1}, and KPC2-Par-1^{KO8} (n=3-4 mice/group) tumor tissue. **** $P < 0.0001$; n.s. - not significant.

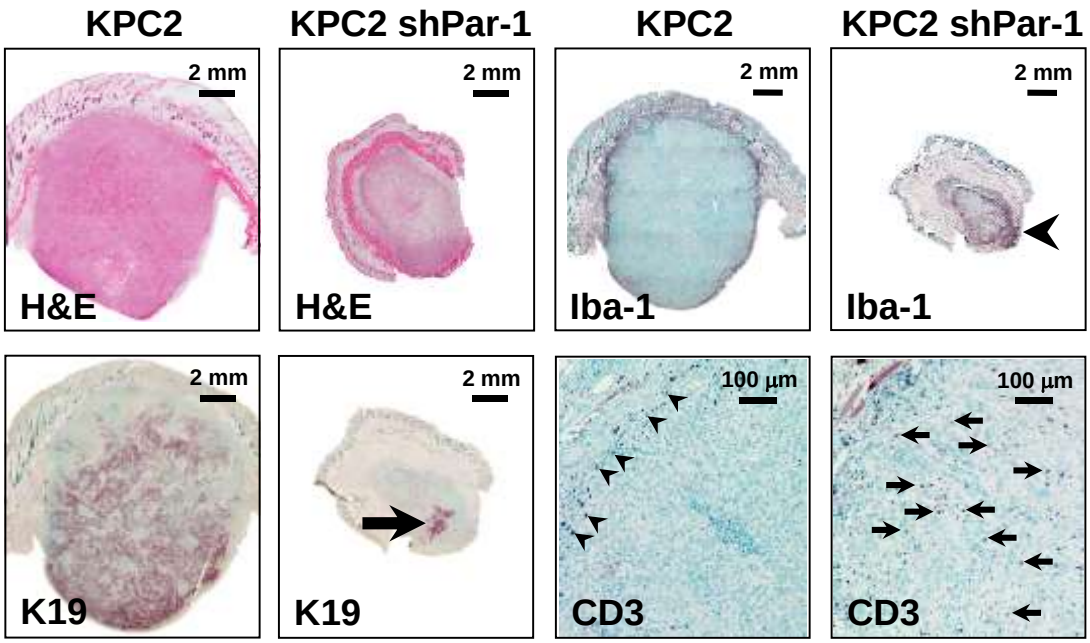

Figure 4.13 Reduction of Par-1 in KPC cells leads to immune cell invasion into tumor tissue. Representative images of tissue sections stained for H&E, cytokeratin 19 (K19), the activated macrophage marker Iba-1, and the T cell marker CD3. The sections were generated from isolated tumor tissue 2 weeks following subcutaneous injection of KPC2 WT and KPC2 shPar-1 cells. Note that arrows and arrowheads highlight the presence of $K19^{+}$, Iba-1^{+} or $CD3^{+}$ cells within the overall tumor mass. Scale bar: 2 mm.

Finally to directly test this hypothesis, we performed parallel tumor growth studies of KPC2-Cas9 cells and KPC2-PAR-1^{KO1} cells in immune competent C57Bl/6 mice and immune-deficient NSG mice. Similar to earlier findings, KPC2-Cas9 cells formed large tumors in C57Bl/6 mice whereas minimal tumor growth of KPC2-PAR-1^{KO1} in C57Bl/6 mice was observed (**Figure 4.14A, B**). In stark contrast, both KPC2-Cas9 and KPC2-PAR-1^{KO1} formed large tumors (equivalent in overall size) in immune-deficient NSG mice (**Figure 4.14A, B**). Collectively, these findings suggest that the failure of tumor growth in immune-competent C57Bl/6 for KPC2-PAR-1^{KO} cells was not due to cell intrinsic alterations in proliferation or apoptosis but rather was linked to aberrant interactions with the TME to suppress anti-tumor immunity.

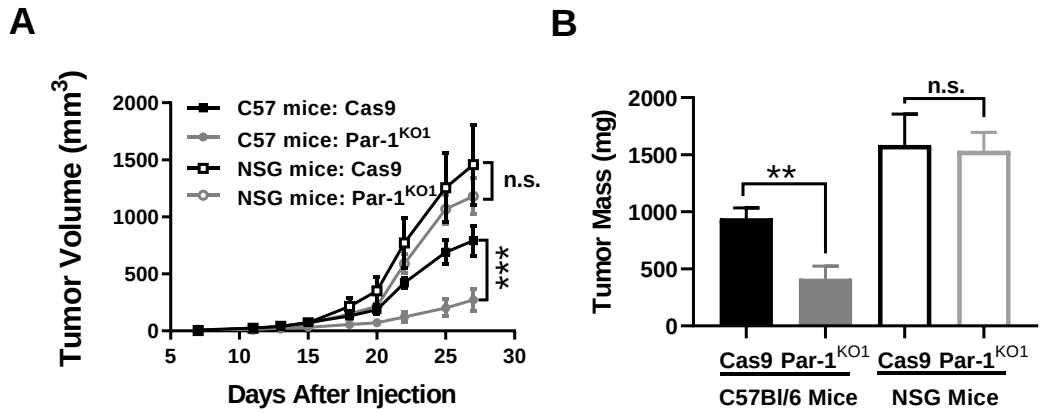

Figure 4.14 Tumor cell thrombin-PAR-1 signaling promotes tumor growth by suppression of anti-tumor immunity. (A) Analysis of tumor volume and **(B)** final tumor mass following subcutaneous injection of KPC2 Cas9 control and KPC2 Par-1^{KO1} cells into cohorts of C57Bl/6 and immune-deficient NSG mice (N=7 mice per group). $^{**}P < 0.01$; $^{***}P < 0.001$; n.s. - not significant.

4.3 Discussion

Thrombin has been linked to cancer progression in a number of contexts, including promoting cell adhesion, enhancing tumor growth and angiogenesis (Nierodzik & Karpatkin, 2006). However, the precise mechanism(s) by which thrombin activation contributes to lethal pancreatic cancer progression has not been elucidated. My studies revealed that tumor cell-derived PAR-1 is a key target of thrombin in that PAR-1 shRNA and CRISPR knockout strategies confirmed that tumor cell PAR-1 is a primary factor in PDAC tumor growth. The reduction in tumor growth in PAR-1 deficient cells was not simply a function of cell-intrinsic defects because *in vitro* proliferation and colony formation in agar were equivalent in KPC cells with and without PAR-1. Importantly, induction of Dox-inducible PAR-1^{Myc} rescued full tumor growth in PAR-1^{KO} cells, confirming a direct dependency of PAR-1 activity by PDAC cells. Tumor PAR-1 was also required for metastasis as PAR-1 deficient cells exhibited a greatly reduced metastatic tumor burden. Together, our studies support a model where PAR-1 is critical for primary tumor growth and formation of metastatic lesions.

The role of PAR-1 in stromal cells has also been shown to contribute to pancreatic cancer progression (Queiroz et al., 2014). Our studies suggest that even in the context of a normal microenvironment with PAR-1 expressing stromal cells of multiple origins (*i.e.*, fibroblasts, inflammatory cells), reduction of thrombin generation in the microenvironment, or elimination of PAR-1 in the tumor cells, is a powerful negative determinant of tumor growth. Interestingly, WT KPC cells readily formed tumors in *F2r*$^{-/-}$ mice indicating that tumor growth was not dependent on the presence of PAR-1 in the TME. However, we have observed reduced growth of KPC shPar-1 cells in *F2r*$^{-/-}$ mice (experiment performed by Dr. Flick), suggesting the existence of a complex synergistic network between PDAC tumor cells and PAR-1 expressing cells within the stromal microenvironment. These preliminary findings highlight the need to better understand the relative

contribution of PAR-1 signaling to the various stages of tumor growth and PDAC disease progression by individual cell types other than tumor cells.

The mechanism by which PAR-1 promotes tumor growth and formation of metastatic lesions has been studied primarily by focusing on the intrinsic cell signaling *in vitro*, such as promoting cell proliferation, migration and adhesion to the cellular matrix (Darmoul et al., 2003, 2004; Even-Ram et al., 2001; Rudroff et al., 2001; Tantivejkul et al., 2005) or by *in vivo* analyses using implanted human cells in immune-deficient mice (Agarwal et al., 2008; Auvergne et al., 2016; Boire et al., 2005; Cisowski et al., 2011; E Yang et al., 2016; Eric Yang et al., 2009). Such strategies do not allow for identifying potential roles of PAR-1 in modulating anti-tumor immune responses. Our strategy, using the KPC system in C57Bl/6 mice, has alleviated this significant limitation. The identification of the Thrombin-PAR-1 axis in immune suppression is consistent with studies showing PDAC has been known to have poor immunogenicity. Recent advances in immune therapies using single-agent immune checkpoint inhibitors targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1(PD-1) have little to no effect on PDAC patients (Brahmer et al., 2012; Royal et al., 2010), indicating that other mechanisms dominantly contribute to PDAC immune suppression. It stands to reason that immune evasion mechanisms for PDAC are associated with the driver mutation genes (*i.e.,* mutations in KRas, TP53) since PDAC has a relatively low mutation rate during cancer progression (Yachida et al., 2010). Accordingly, the driver mutation genes would confer upon the PDAC cancer cell an immune escape capacity at the earliest stages of PDAC disease. The identification of TF and PAR-1 genes as downstream targets of $KRas^{G12D}$ and $P53^{R172H}$ are consistent with this working model. In support of this concept, the dynamics of PDAC TME progression where proinflammatory immune components (CD8 T cells, T helper cells and dendritic cells) has been observed in early

stage PDAC and replaced afterwards by immunosuppressive myeloid-derived tumor suppression cells (MDSCs) at late stages (Bernard et al., 2018) could provide an explanation for why eliminating tumor cell PAR-1 expression at early (small size) tumor stages results in tumor regression but no effect when tumors are large (**Figure 4.10**). PAR-1 may be responsible for creating the immune suppressive environment by suppressing the proinflammatory immune components and recruiting anti-inflammatory MDSCs. Once MDSCs have established, removing PAR-1 would have limited effect on tumor immunity. Indeed, thrombosis mediated pathways (TF, PAR-1) have been shown to recruit MDSCs and suppress the immune system (Han et al., 2017; Queiroz et al., 2014). PAR-1 has also been shown to alter immune responses directly in other contexts, including inflammatory disease and response to viral infection (Antoniak et al., 2013, 2017; Khoufache et al., 2013; Saban et al., 2007; Steinhoff et al., 2005). Although current data strongly support a role for tumor cell-derived PAR-1 as an immune modulator in the PDAC TME, critical future experiments will focus on identifying the various components of the immune system impacted (*e.g.*, immune effector cell types) and the specific gene products downstream of PAR-1 signaling molecules that connect to the immune system.

CHAPTER 5. SUMMARY AND FUTURE DIRECTIONS

PDAC is among the most lethal malignancies with a 8% 5-year survival rate (Siegel et al., 2018). The dismal survival is mainly due to ineffective chemo- and radiotherapies which are at least in part, due to (i) a heterogeneous TME that forms a hypovascular and immunosuppressive environment and (ii) a lack of consistent early detection mechanisms. Together, these two issues highlight the urgency to understand the early development of PDAC, especially the early dynamics of how the TME interacts with tumor cells. An interesting phenomenon of PDAC patients is that they often suffer thrombosis-related complications. However, the molecular mechanistic connections between pancreatic cancer and thrombosis have not been elucidated despite a number of correlation studies showing a link between key components within the coagulation cascade (e.g., TF and PAR-1) and PDAC aggressiveness or patient survival. By using GEMM and multiple genetic approaches, my present study provides the first clear connection between thrombosis in the TME and PDAC progression. From these studies we have proposed a central model were pancreas acinar cells that acquire genetic mutations ($KRas^{G12D}$ and $P53^{R172H}$) transform into PDAC cancer cells and up-regulate TF and PAR-1 expression. Cell surface TF (or secreted TF contained within macro particles) react with circulating prothrombin to produce a thrombin enriched TME. Thrombin subsequently activates PAR-1 on the cancer cell surface which induces downstream G protein coupled signaling pathways, leading to suppression of anti-tumor immunity and promoting PDAC tumor growth and metastasis (**Figure 5.1**).

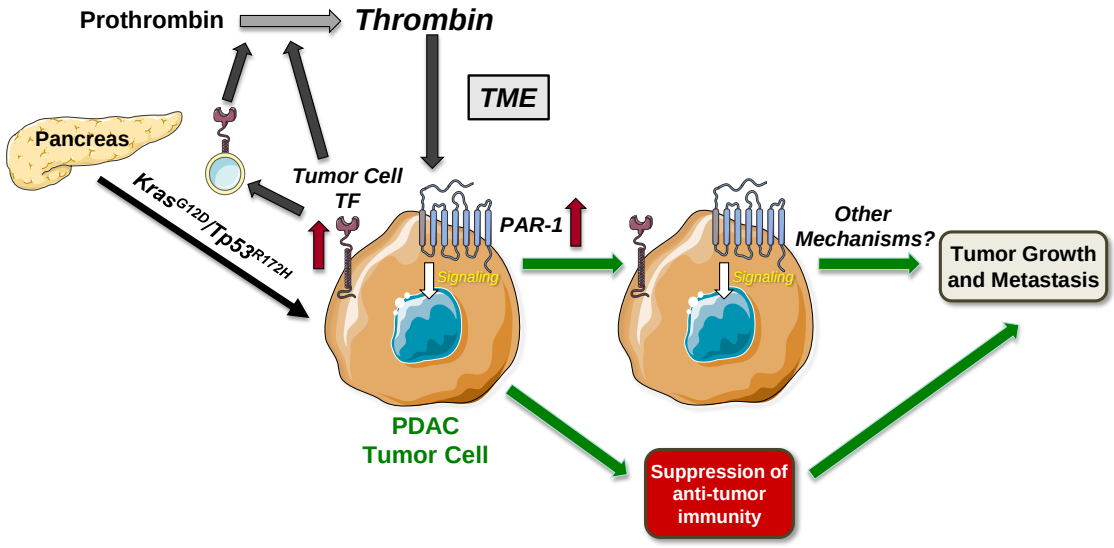

Figure 5.1 Final model of the TF-Thrombin-PAR-1 axis in pancreatic cancer pathogenesis. $KRas^{G12D}/P53^{R172H}$-induced transformation leads to upregulation of TF which induces conversion of prothrombin to thrombin in the microenvironment. Thrombin subsequently activates PAR-1 signaling on the tumor cell, initiating downstream events that support primary PDAC tumor growth and metastasis, likely through suppression of anti-tumor immunity.

In 1993, Vogelstein and Kinzler purposed a general model for cancer progression entitled "Multistep Nature of Cancer" where several mutations are required for cancer to progress and each subsequent mutation drives a series of cellular changes associated with a gradual increase in tumor aggressiveness and invasiveness (Vogelstein & Kinzler, 1993). Later in 2000, Ralph Hruban adopted this model and purposed a rational progression model for pancreatic cancer by adding specific pathological and genetic information (Hruban et al., 2000) (**Figure 1.2**). However, such a model does not include information on pancreatic cancer metastasis which is a major determinant of patient mortality. Recent mouse genetic and lineage tracing studies have shown that pancreatic cancer cells disseminate early in the disease even before the primary tumor can be detected by histologic analysis. More importantly, metastatic tumors at distant organs lack additional driver mutations when compared to the respective primary tumors, which is inconsistent with the

multistep progression model for most other cancers (Haeno et al., 2012; Makohon-Moore et al., 2017; Rhim et al., 2012). Indeed, these discoveries are in agreement with the poor prognosis of post-surgery PDAC patients where no microscopic tumor remains at the primary tumor site or at nearby lymph nodes (R0 and N0 nodal). In these incidences the 5-year survival rate is less than 21%, mainly due to metastatic disease (Sobin et al., 1988). When combined with known genetic information, we can speculate that the primary mutations in PDAC are sufficient to drive cancer cells towards robust tumor growth but also to disseminate from the primary tumor. Given that KRas and p53 mutations directly induce cancer cell TF and PAR-1 expression, and my study has provided substantial evidence that cancer cell derived TF and PAR-1 promote metastasis and tumor growth, our current data fit this model very well. On the other hand, the novel part of my study is that PAR-1 downstream signaling promotes PDAC tumor growth in part by mediating cancer cell immune evasion, possibly through mechanisms where PAR-1 reshapes the TME from a highly immunogenic environment to a poor immunogenic environment at early stages of PDAC (**Figure 4.13**), ultimately aiding tumor cells to escape the anti-tumor immunity. This connection links two unexpected features of pancreatic cancer: a hyperactive thrombosis cascade and poor immunogenicity. Future studies will focus on the following key questions:

i) What are the specific downstream molecular mechanisms by which PAR-1 promotes PDAC immune evasion?

In order to fully dissect the molecular pathways downstream of PAR-1, the following sub-goals need to be achieved. (a) Determine which immune cell components are responsible for suppressing Par-1^{KO1} cell tumor growth. These studies should utilize different immune genetic mouse models ($Rag1^{-/-}$; CD11b-DTR mouse models) or antibody depletion assays in combination with our allograft models to access the tumor growth restoration (outside the cell membrane); (b)

Define the cellular localization of effective PAR-1 downstream factors (secreted proteins or membrane localized proteins). For these studies it would be good to generate KPC2-GFP and Par-1^{KO1}-RFP cells and co-inject the cells into mice and determine the survival of KPC2 and Par-1^{KO1} cells *in vivo*. Based on the result of counting cell number inside the tumor, one can differentiate whether the effective factor is localized on the cell membrane (low cell number Par-1^{KO1} cells and high cell number of KPC2 cells) or secreted outside the cell membrane (equal numbers of Par-1^{KO1} and KPC2 cells); (c) Identify the changes in expression of gene sets in response to thrombin. RNA sequencing using KPC2, PAR-1^{KO1} and PAR-1^{KO8} cells ± thrombin will provide a wealth of information which will assist in identifying further key players to eventually reveal the final molecular mechanism (**Figure 5.2**).

ii) *What role does the TF-thrombin-PAR-1 pathway play at early endogenous PDAC disease (i.e., initial events such as acinar-ductal metaplasia or early development)?*

For this study, KPC mice should be generated that are homozygous for an *F2r*$^{fl/fl}$ allele (KPC PAR-1^{cKO} mice). Wildtype, KPC and KPC PAR-1^{cKO} mice can be generated by standard crossing to induce expression of the mutant *Kras*^{G12D} and *Trp53*^{R172H} alleles and spontaneously delete *F2r* in pancreatic acinar cells. Under normal conditions, KPC mice develop pre-neoplastic PanIN lesions by 3 months. However, our expectation is that the KPC Par-1^{cKO} mice will be refractile to developing PanINs and PDAC owing to deletion of the *F2r* gene.

Taken together, by utilizing multiple genetic and pharmacological approaches, I discovered that the TF-Thrombin-PAR-1 axis promotes tumor growth and metastasis *in vivo*. In addition, PAR-1 is the gateway of PDAC tumor growth and metastasis. More importantly, PAR-1 promotes PDAC tumor growth in part through suppressing anti-tumor immunity *in vivo*. My results offer the proof-of-principle that targeting thrombin and/or tumor cell PAR-1 may offer substantial therapeutic benefits to PDAC patients.

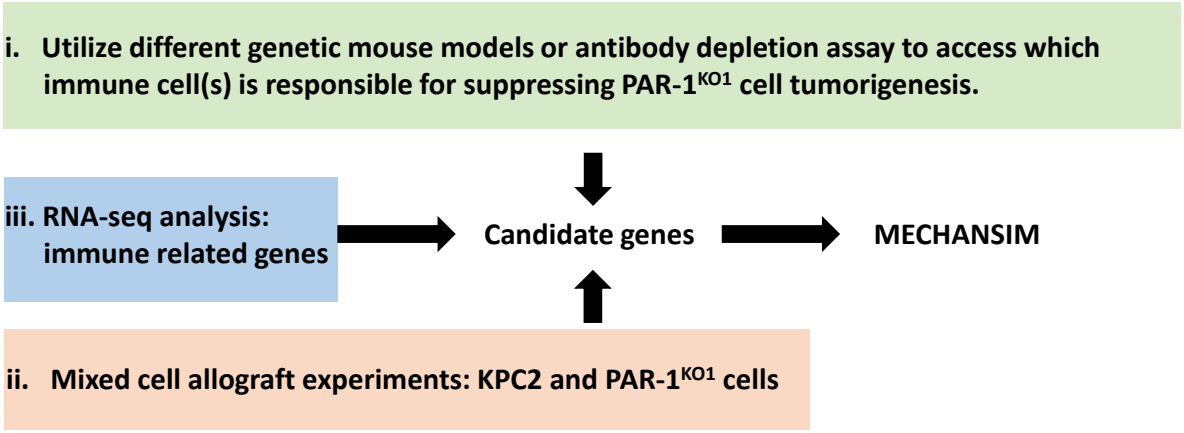

Figure 5.2 Proposed strategy to investigate PAR-1 downstream molecular mechanisms. (i) Accessing the specific immune components that are regulating PAR-1 mediated immune suppression; (ii) Narrowing down a potential gene list by mixed cell allografts to determine the essential gene localization, and (iii) Performing RNA-seq analysis to obtain differentially expressed genes of KPC cells upon thrombin treatment.

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VITA

Yi graduated from No.1 middle school affiliated to Central China Normal University in 2006 and got his Bachelor's degree with a major in biotechnology from Wuhan Institute of Technology in China in 2010. He then entered the United States and obtained his Master's degree in biochemistry and molecular biology from George Washington University with a research focus on DNA damage and repair. Afterwards, Yi was enrolled in the Interdisciplinary Life Science Program (PULSe) at Purdue University in West Lafayette in the fall of 2012 and joined Dr. Stephen Konieczny's lab in the summer of 2013. Yi's primary research focused on coagulation cascade signaling in pancreatic ductal adenocarcinoma. Yi earned his Ph.D. degree in May, 2019 and he has accepted a postdoctoral position in the department of pathology and laboratory medicine, University of North Carolina, Chapel Hill. Yi will work with Dr. Matthew Flick to continue studying thrombosis and the pancreatic cancer tumor microenvironment.

PUBLICATIONS

1. **Yang Y**, Stang A, Schweickert PG, Lanman, NA, Paul E, Monia BP, Revenko AS, Palumbo JS, Mullins ES, Elzey BD, Janssen EM, Konieczny SF, Flick MJ. Thrombin Signaling Promotes Pancreatic Adenocarcinoma through PAR-1 Dependent Immune Evasion. *Cancer Research*, 2019 In Press.

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