

A PK2/Bv8 Antagonist Suppresses Tumorigenic Processes by Inhibiting  
Angiogenesis in Glioma and Blocking Myeloid Cell Infiltration in Pancreatic Cancer

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Dissertation submitted in partial fulfillment of  
the requirements for the degree of Doctor of Philosophy in the Department of  
Pharmacology and Cancer Biology in the Graduate School  
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ABSTRACT

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## Abstract

In many cancer types, infiltration of bone marrow-derived myeloid cells in the tumor microenvironment is often associated with enhanced angiogenesis and tumor progression, resulting in poor prognosis. The polypeptide chemokine PK2 (Bv8) regulates myeloid cell mobilization from the bone marrow, leading to activation of angiogenesis as well as accumulation of macrophages and neutrophils in the tumor site. Neutralizing antibodies against PK2 display potent anti-tumor efficacy, illustrating the potential of PK2-antagonists as therapeutic agents for the treatment of cancer. However, antibody-based therapies can be too large to treat certain diseases and too expensive to manufacture while small molecule therapeutics are not prohibitive in these ways. In this study, we demonstrate the anti-tumor activity of a small molecule PK2 antagonist, PKRA7, in the contexts of glioblastoma and pancreatic cancer xenograft tumor models. In the highly vascularized glioblastoma, PKRA7 decreased blood vessel density while increasing necrotic areas in the tumor mass. Consistent with the anti-angiogenic activity of PKRA7 *in vivo*, this compound effectively reduced PK2-induced microvascular endothelial cell branching *in vitro*. For the poorly vascularized pancreatic cancer, the primary anti-tumor effect of PKRA7 is mediated by the blockage of myeloid cell migration and infiltration. At the molecular level, PKRA7 inhibits PK2-induced expression of several pro-migratory chemokines and chemokine receptors in macrophages. Combining PKRA7 treatment with standard chemotherapeutic agents resulted in enhanced effects in xenograft models for both glioblastoma and pancreatic tumors. Taken together, our

results indicate that the anti-tumor activity of PKRA7 can be mediated by distinct mechanisms that are relevant to the pathological features of the specific type of cancer. This small molecule PK2 antagonist holds the promise to be further developed as an effective agent for combinational cancer therapy.

## **Dedication**

This dissertation is dedicated to my grandmothers, Mrs. Anne Curtis and Mrs. Janine Forbes who I know would be extremely proud of their granddaughter if they were here today. Thank you for all the love and support!

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## **List of Abbreviations**

5-FU – fluorouracil

ACE – adrenal cortical capillary endothelial cell

AFP – alpha-fetoprotein

BBB – blood brain barrier

Bcl2L12 – Bcl2-like 12

bFGF – basic fibroblast growth factor

BMDC – bone marrow-derived cell

CAF – cancer-associated fibroblasts

CHO – Chinese hamster ovary

COX-2 – cyclooxygenase-2

CRC – colorectal cancer

CSC – cancer stem cells

CSF-1 – colony-stimulating factor 1

CTL – cytotoxic T lymphocyte

dCTP – deoxycytidine triphosphate

DNA – deoxyribonucleic acid

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

EpCAM – epithelial cell adhesion molecule

FDA – Food and Drug Administration

FGF – fibroblast growth factor

GBM – glioblastoma multiforme/glioblastoma

G-CSF – granulocyte colony-stimulating factor

GDP – guanosine-diphosphate

GM-CSF – granulocyte-macrophage colony-stimulating factor

GEM – gemcitabine

GI – gastrointestinal

GPCR – G-protein-coupled receptor

GTP – guanosine-triphosphate

HCC – hepatocellular carcinoma

HIF-1 $\alpha$  – hypoxia-inducible factor 1 alpha

HMEC-1 – human microvascular endothelial cell 1

IC – intracranial

IGF – insulin-like growth factor

IHC – immunohistochemistry

IHMVEC – immortalized human microvascular endothelial cell

IL-3 – interleukin 3

IL-4 – interleukin 4

IL-6 – interleukin 6

IL-8 – interleukin 8

IL-10 – interleukin 10

IL-12 – interleukin 12

IL-13 – interleukin 13

INF $\gamma$  – interferon gamma  
IP – intraperitoneal  
IR – ionizing radiation  
LIF – leukemia inhibitory factor  
LLC – Lewis lung carcinoma  
MAPK – mitogen-activated protein kinase  
MCP-1 – monocyte chemoattractant protein 1  
MDM2 – murine double minute 2  
MDSC – myeloid-derived suppressor cell  
MEEC – mouse embryonic endothelial cell  
MGMT – O6-methylguanine-DNA methyltransferase  
MHC – major histocompatibility complex  
MMP-9 – matrix metalloproteinase 9  
MRI – magnetic resonance imaging  
mRNA – messenger RNA  
Ndr $g_1$  – N-myc downstream regulated gene 1  
NF $\kappa$ B – nuclear factor kappa-light-chain-enhancer of activated B cells  
NK – natural killer cell  
NO – nitric oxide  
NSCLC – non-small cell lung cancer  
OSM – oncostatin-M  
PanIN – pancreatic intraepithelial neoplasia

PBS – phosphate-buffered saline

PDA – pancreatic ductal adenocarcinoma

PDGF – platelet-derived growth factor

PGE<sub>2</sub> – prostaglandin E<sub>2</sub>

PI3K – phosphatidylinositol 3'-kinase

PIGF – placental growth factor

PK1 – prokineticin 1

PK2 – prokineticin 2

PKR1 – prokineticin receptor 1

PKR2 – prokineticin receptor 2

PKRA7 – prokineticin receptor antagonist 7

PTEN – phosphatase and tensin homolog

PVTT – portal vein tumor thrombus

RCC – renal cell carcinoma

RIP-Tag – rat insulin promoter-T antigen

RNA – ribonucleic acid

ROS – reactive oxygen species

RTK – receptor tyrosine kinase

SC – subcutaneous

SCN – suprachiasmatic nucleus

SDF-1 – stromal cell-derived factor-1

shRNA – short hairpin RNA

STAT3 – signal transducer and activator of transcription 3

TAM – tumor-associated macrophage

TGF- $\beta$  – transforming growth factor beta

Th1 – T helper 1 cell

Th2 – T helper 2 cell

TMZ – temozolomide

TNF $\alpha$  – tumor necrosis factor alpha

T<sub>reg</sub> – regulatory T cell

VEGF – vascular endothelial growth factor

VEGFR – vascular endothelial growth factor receptor

WHO – World Health Organization

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# **1. Introduction**

## ***1.1 The Tumor Microenvironment Contributes to Cancer Progression***

Cancer involves an intricate assortment of heterogeneous cells and not a mass of clonal tumor cells as was previously thought. All the different cell types combined create the tumor and the tumor microenvironment. The complex tumor microenvironment is an important contributor to tumorigenesis through processes such as angiogenesis, immunosuppression and tumor cell motility [Kusmartsev and Gabrilovich, 2006; Pollard, 2004]. Much attention has been placed on understanding how and why tumor cells proliferate and most current therapeutics directly target the growing tumor cells. However, in recent years, increased focus has been placed on targeting the stromal cells in the tumor microenvironment that are responsible for various aspects of the tumorigenic process. Stromal cells in the tumor microenvironment include cancer-associated fibroblasts (CAFs), endothelial cells, pericytes, immune inflammatory cells and local and bone marrow-derived stromal stem and progenitor cells [Hanahan and Weinberg, 2011].

### **1.1.1 Myeloid Cells in the Tumor Microenvironment**

Bone marrow-derived myeloid cells, which are precursors to macrophages, neutrophils and myeloid-derived suppressor cells, represent a subpopulation of stromal cells that play important roles during tumor progression [Shojaei *et al.*,

2008b]. In response to cytokines/chemokines secreted by tumor cells, myeloid cells can be mobilized from the bone marrow and infiltrate into tumor sites where they can promote growth, invasion and angiogenesis to support tumor expansion and metastasis [Kusmartsev and Gabrilovich, 2006; Pollard, 2004]. For instance, granulocyte colony stimulating factor (G-CSF) produced by tumor cells can lead to the differentiation of CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells into neutrophils, macrophages, and dendritic cells that are found at high levels in the tumors of cancer patients and tumor-bearing mice [Diaz-Montero *et al.*, 2009; Shojaei *et al.*, 2008b; Yang *et al.*, 2004]. Macrophage differentiation, growth and chemotaxis can be regulated by several factors produced by cancer cells including colony-stimulating factor 1 (CSF-1), granulocyte-macrophage (GM)-CSF, interleukin 3 (IL-3) and CCL2, all of which can recruit macrophages to the tumor site [Pollard, 2009]. Indeed, high numbers of tumor-associated macrophages (TAMs) in various cancers such as breast, prostate, ovarian, cervical, bladder, kidney, esophageal, and squamous cell carcinoma are associated with poor prognosis for these diseases [Lewis and Pollard, 2006; Pollard, 2004].

While myeloid progenitor cells and their progeny such as neutrophils, dendritic cells and macrophages have been studied in the tumor microenvironment, many studies have specifically emphasized the contribution of macrophages and macrophage subsets. Studies have found that, overall, TAMs shift towards a tumor-promoting subset population to support tumor growth through a variety of mechanisms such as enhancing tumor cell proliferation and invasion, and

contributing to angiogenesis, immunosuppression and metastasis [Qian and Pollard, 2010].

#### **1.1.1.1 The Role of Myeloid Cells in Tumor Initiation**

The “root cause” of some cancers is thought to be persistent or chronic inflammation due to other illness or diseases [Qian and Pollard, 2010]. If the negative controls of the immune system become compromised the inflammatory cells can create a pro-tumorigenic environment. Two factors present in myeloid cells, NF $\kappa$ B and STAT3, normally work in opposition to maintain this balance. NF $\kappa$ B is a signal transducer of inflammation, causing downstream activation of Toll-like receptors (TLRs) and the expression of pro-inflammatory factors such as interleukin 12 (IL-12) and tumor necrosis factor alpha (TNF $\alpha$ ). STAT3, however, suppresses the inflammatory response as it is a target of interleukin 10 (IL-10), an immunosuppressive cytokine. Immune cells present at chronic sites of inflammation are thought to create a mutagenic microenvironment as reactive oxygen species (ROS) and nitric oxide (NO) produced by the immune cells can create mutations and genetic instability in nearby epithelial cells. These mutations can be perpetuated as the epithelial cells respond to growth factors secreted by the macrophages present at the site of inflammation and this may ultimately lead to cancer initiation [Qian and Pollard, 2010]. While not all cancers arise from chronic inflammation, there is evidence that macrophages present in the tumor microenvironments of a variety of cancer types can contribute to tumor progression.

### 1.1.1.2 Myeloid Cells Can Enhance Tumor Cell Proliferation

Macrophages can be classified into two subsets based on differential immunological responses. Within the first subset are “activated” macrophages, also referred to as M1 macrophages and they are involved in the responses of type I helper T (Th1) cells to pathogens [Qian and Pollard, 2010]. These M1 macrophages are activated by interferon gamma ( $\text{INF}\gamma$ ) and TLR signaling and express major histocompatibility complex (MHC) class II, IL-12 and  $\text{TNF}\alpha$ . These factors and the generation of ROS and NO give the M1 macrophages the ability to kill pathogens and cells. In contrast, the second subset of macrophages are said to be “alternatively activated” and are referred to as M2 macrophages. These M2 macrophages result from responses to interleukin 4 (IL-4), IL-10 and interleukin 13 (IL-13) and are involved in type II helper T (Th2) cell responses like wound healing, promoting angiogenesis and tissue remodeling [Gordon, 2003; Kurahara *et al.*, 2011]. Macrophages present at sites of inflammation where tumor initiation has occurred or those that have been recruited to tumor initiation sites are thought to transition from the “activated” M1 phenotype to the “alternatively activated” M2 phenotype and contribute to tumor progression through their pro-tumoral functions [Pollard, 2009]. This transition is aided by tumors constitutively expressing p50 homodimers that inhibit pro-inflammatory  $\text{NF}\kappa\text{B}$  signaling. These M2 macrophages can now support tumor growth in various ways, including directly contributing to tumor cells proliferation [Lewis and Pollard, 2006].

Various studies have shown that the presence of macrophages in the tumor microenvironment correlates with tumor cell proliferation [Lewis and Pollard, 2006]. These TAMs can express a variety of factors that can stimulate tumor cell proliferation and survival such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- $\beta$ ), hepatocyte growth factor (HGF) and basic fibroblast growth factor (bFGF). Co-culture experiments with tumor cells and macrophages indicate that macrophages secrete factors that stimulate the proliferation of tumor cells. This proliferation is important for tumor progression but TAMs support many other processes that lead to increased malignancy.

#### **1.1.1.3 Myeloid Cells are Involved in the Invasion of Tumor Cells into the Surrounding Normal Tissue**

The presence of TAMs in the tumor microenvironment can affect the invasive capabilities of tumor cells by activating invasive signaling pathways in tumor cells and contributing to the breakdown of the surrounding stroma [Lewis and Pollard, 2006; Qian and Pollard, 2010]. CSF-1 expressed by tumor cells recruits macrophages and can lead to the expression of EGF by the macrophages. This EGF can then activate the migration of the tumor cells [Wyckoff *et al.*, 2004]. TAMs can also upregulate proteolytic enzymes such as cathepsin D, matrix metalloproteinases (MMPs) and serine proteases to breakdown the basement membrane, making it easier for the migrating tumor cells to escape [Domagala *et al.*, 1992; Qian and

Pollard, 2010]. In models of breast cancer, IL-4 expression by mature CD4<sup>+</sup> helper T cells or tumor cells can direct the polarization of macrophages to an invasion-promoting phenotype while expression of stromal cell-derived factor 1 (SDF-1) can also support tumor cell and macrophage comigration [Qian and Pollard, 2010].

#### **1.1.1.4 Myeloid Cells are Important Contributors to Tumor Angiogenesis**

The expansion of a growing tumor creates the need for additional oxygen and nutrient supplies to support the tumor cells which must be met through angiogenesis, the growth of new blood vessels from pre-existing ones. There is a correlation between increased number of TAMs and high vascular grades in breast cancer, malignant uveal melanoma, glioma, bladder cancer, prostate cancer and others [Lewis and Pollard, 2006]. Further studies have shown that macrophages are vital to angiogenesis as TAMs express many proangiogenic factors such as vascular endothelial growth factor (VEGF), TNF $\alpha$ , interleukin 8 (IL-8), bFGF, cyclooxygenase-2 (COX-2) and angiopoietin [Lewis and Pollard, 2006]. VEGF is a centrally important protein involved in angiogenesis that promotes blood vessel growth through binding to VEGF receptors (VEGFRs) on endothelial cells. This interaction activates downstream pathways such as phosphatidylinositol 3'-kinase (PI3K)/Akt and Raf1/MEK/mitogen-activated protein kinase (MAPK) that drive angiogenesis and enhance vascular integrity [Kerbel, 2008]. TAMs can directly express and secrete VEGF or they can make it available by expressing matrix metalloproteinase 9 (MMP9) which can release VEGF from the extracellular matrix [Du *et al.*, 2008].

Macrophages can also be recruited to hypoxic/necrotic areas of tumors through CCL2 and endothelin chemokine expression. Expression of the transcription factor hypoxia inducible factor alpha (HIF1 $\alpha$ ) is constitutive in macrophages, leading to VEGF expression and revascularization of the hypoxic region [Murdoch *et al.*, 2008]. The expression of the angiopoietin receptor Tie2 on macrophages and monocytes can also support tumor angiogenesis, and ablation of these cells specifically has been shown to reduce angiogenesis and lead to tumor regression in a glioma xenograft model [De Palma *et al.*, 2005].

#### **1.1.1.5 Myeloid Cells Contribute to Immunosuppression in the Tumor Microenvironment**

TAMs have poor antigen-presenting capabilities and suppress tumor-antagonizing CD8<sup>+</sup> cytotoxic T lymphocytes (CTL) and natural killer (NK) cell proliferation and activity, further promoting tumor progression through immunosuppression [Lewis and Pollard, 2006; Ostrand-Rosenberg and Sinha, 2009; Qian and Pollard, 2010]. Therefore, at the tumor site, myeloid cell recruitment can contribute to tumor expansion by directly contributing to tumor proliferation and angiogenesis as well as diminishing the immune response [Hanahan and Weinberg, 2011]. TAMs in the tumor microenvironment are often found to have lost the ability to present tumor-associated antigens to T cells and NK cells, reducing the antitumor activity of these cytotoxic cells and allowing the tumors to escape the immune response [Lewis and Pollard, 2006]. This immunosuppression is mediated through a

variety of factors and pathways. Tumor-promoting macrophages downregulate pro-immune response genes such as IL-12, IL-18 and the TLR signaling pathway [Qian and Pollard, 2010]. Additionally, tumor cells can secrete factors such as IL-4, interleukin 6 (IL-6), IL-10, TGF- $\beta$  and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) to inhibit the cytotoxic activity of TAMs [Lewis and Pollard, 2006]. TGF- $\beta$ , IL-10 and PGE<sub>2</sub> expressed by the tumor cells may suppress the expression of MHC class II molecules by macrophages which reduces their ability to present tumor-associated antigens to T cells [Elgert *et al.*, 1998]. These factors can also reduce the expression of IL-12 which is involved in the proliferation and cytotoxicity of T cells and NK cells [Lewis and Pollard, 2006]. Tumor cells work with TAMs to reduce the presence and activity of the cytotoxic cells of the immune system. This allows the tumor cells to escape detection and continue to proliferate without interference from immune cells.

#### **1.1.1.6 Myeloid Cells are Involved in the Regulation of Metastasis**

TAMs can support a tumor's growth through many processes and this support can enable the release of tumor cells from the primary tumor and the establishment of secondary tumors at distant sites called metastases. Indeed, increased numbers of TAMs in primary tumors correlates with early establishment of metastases in breast cancer and bladder cancer [Hanada *et al.*, 2000; Leek *et al.*, 1996].

Macrophages can be recruited to premetastatic niches through tumor-produced myeloid cell chemoattractants such as S100A8 and S100A9 to prime the site for tumor cells [Kaplan *et al.*, 2005]. TAMs are also thought to contribute to tumor cell

extravasation from blood vessels into metastatic sites as clusters of macrophages have been observed attached to the abluminal side of vessels [Wyckoff *et al.*, 2004]. Mouse models of cancer have further demonstrated a role for macrophages in promoting metastasis. Lin *et al.* observed a decrease in lung metastasis in a mouse model of PyMT-induced mammary tumors when macrophages were systemically depleted [Lin *et al.*, 2001]. Additionally, orthotopic injection of colon cancer cells into metastatic sites such as the peritoneum or liver portal vein formed tumors more slowly in mice where macrophages were selectively depleted in the peritoneal cavity or the liver compared to mice with an intact macrophage population [Oosterling *et al.*, 2005]. Clinical research has demonstrated a correlation between an increased number of macrophages in lymph node metastases and poor patient survival, giving further evidence to the concept of macrophages supporting tumor growth at metastatic sites [Oberg *et al.*, 2002].

### **1.1.2 The Unique Tumor Microenvironments of Glioblastoma and Pancreatic Cancer**

All cancer types have unique tumor microenvironments that become individually tailored to the specific tumor cells to further their expansion [Lewis and Pollard, 2006]. We are interested in two cancer types with very different tumor microenvironment compositions, glioblastoma multiforme (GBM) and pancreatic cancer. GBM is highly vascularized, whereas pancreatic cancer is often poorly vascularized but highly fibrotic with a large portion of the tumor mass consisting of

stromal components including infiltrated macrophages [de Groot *et al.*, 2010; Korc, 2007; Norden *et al.*, 2008]. Both GBM and pancreatic cancer have extremely poor prognoses due to high frequency of late stage diagnoses and the lack of effective therapies. One common feature of the tumor microenvironments of both GBM and pancreatic cancer is the involvement of myeloid cells [Badie and Schartner, 2000; Kurahara *et al.*, 2011; Roggendorf *et al.*, 1996].

#### 1.1.2.1 Glioblastoma

GBM, World Health Organization (WHO) grade IV, is the most common and biologically aggressive malignant glioma and is defined by extensive cellular proliferation, diffuse infiltration, robust angiogenesis, and resistance to apoptosis [Furnari *et al.*, 2007]. Intratumoral heterogeneity and a putative cancer stem cell (CSC) subpopulation have made GBM a particularly difficult cancer to understand and treat. As such, the median survival of GBM remains at 12 months over the last decade, even with the therapeutics that have been recently developed. The diffuse nature of GBM makes complete surgical resection almost impossible without damaging normal brain tissue, leaving behind cancerous tissue. Subsequent radiotherapy can kill some of the remaining cancerous tissue, but CD133<sup>+</sup> CSCs are radioresistant and capable of repopulating the tumor, and thus recurrence is very frequent [Bao *et al.*, 2006; Stupp *et al.*, 2005]. Further complicating matters, there are two subtypes of GBM, primary (de novo) and secondary (progressive). The majority of GBM cases are primary GBM and usually occur in older patients

compared to secondary GBM which is mostly seen in patients 45 years old and younger.

While both GBM types have similar pathobiology and median survival rates, their presentation and genetic mutations differ. Secondary GBMs are derived from lower grade astrocytomas that develop and progress into GBM 5-10 years after initial diagnosis, and may be characterized by 10q loss and *p53* or *phosphatase and tensin homolog (PTEN)* mutations [Furnari *et al.*, 2007; Louis, 2006]. In contrast, primary GBMs present with no evidence of prior symptoms while common genetic alterations include *p16<sup>INK4a</sup>p14<sup>ARF</sup>* mutations, *epidermal growth factor receptor (EGFR)* amplifications or mutations, *cyclin D1/3* and *murine double minute 2 (MDM2)* amplifications and *PTEN* mutations [Furnari *et al.*, 2007; Louis, 2006].

Despite extensive research on the molecular pathologies and signatures of GBM, effective therapies targeting these mutations have been elusive. One chemotherapeutic drug, temozolomide (TMZ) has emerged as a viable treatment option. TMZ is a derivative of imidazotetrazinone that is completely absorbed after oral administration and spontaneously converts to its active metabolite [Stupp *et al.*, 2001]. This active metabolite inactivates the DNA repair enzyme O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT), and lower levels of MGMT in tumor tissue have been associated with longer survival [Stupp *et al.*, 2005]. Clinical trials with TMZ alone or combined with radiotherapy has demonstrated significant survival benefits and TMZ has become the standard of care for patients with GBM [Stupp *et al.*, 2005; Yung *et al.*, 2000].

Necrosis is a hallmark feature of GBM, the most prominent form of spontaneous cell death in GBM and a powerful predictor of poor prognosis [Furnari *et al.*, 2007; Louis, 2006]. In contrast to apoptosis, during which cells undergo programmed cell death and are neatly consumed by surrounding cells, necrosis results in cells becoming bloated and exploding, releasing their cellular contents into the surrounding tissue [Hanahan and Weinberg, 2011]. It is thought that necrosis arises in GBM due to a number of factors. Small regions of necrosis may become present in areas where the metabolic need exceeds the supply of oxygen and nutrients, while larger areas of necrosis may develop due to vascular thrombosis blocking the blood supply to the tumors [Louis, 2006]. Other theories point to the hypoxic nature of necrotic centers and the observation of “pseudopalisades” or perinecrotic tumor cells that have migrated away from the central region of the tumor and now surround the necrotic center of the tumor. These cells express HIF-1 $\alpha$ , a transcription factor that facilitates a cell’s survival under hypoxic conditions [Brat *et al.*, 2004]. Hypoxia may further contribute to tumor progression through the release of growth factors by dying cells or permitting the emergence of resistant clones of highly malignant tumor cells. Overexpression of inhibitors of apoptosis in GBM, such as Bcl2-like 12 (Bcl2L12), may also contribute to necrosis as the programmed cell death pathway, apoptosis, may be blocked thus switching cells to necrotic cell death [Furnari *et al.*, 2007]. Linked with necrosis and hypoxia is the process of angiogenesis, the growth of new blood vessels from pre-existing ones to provide the

blood supply that necrotic/hypoxic tumor tissue needs to continue to survive.

Angiogenesis is therefore another important hallmark of GBM.

GBM is one of the most highly vascular solid tumors. Proliferating endothelial cells, observed as microvascular hyperplasia in both primary and secondary GBMs, are a defining phenotype of these tumors [Furnari *et al.*, 2007]. This increase in microvascular proliferation is observed in the transition from low grade or anaplastic astrocytomas to secondary GBM. Numerous pathways such as HIF, VEGF and PDGF converge to drive angiogenesis in GBM [Furnari *et al.*, 2007; Louis, 2006]. Though increased angiogenesis in GBM may be triggered by necrosis/hypoxia, there are a number of glioma-relevant mutations in genes such as *PTEN*, *EGFR* and *CMYC* that often act as an “angiogenic switch”, stabilizing HIF-1 $\alpha$  or its downstream target VEGF even in the absence of canonical angiogenic pathway signaling [Furnari *et al.*, 2007]. The resulting microvessels in the tumor are usually highly tortuous and have a decreased gradient for oxygen delivery, providing little oxygen/nutrient support to the tumor. Rather, the microvessels paradoxically further exacerbate the unbalanced supply of nutrients and oxygen by improperly distributing the blood flow, resulting in further hypoxia and necrosis, thus contributing to a vicious cycle that defines GBM.

The presence of infiltrative inflammatory cells is a feature of GBM that is less understood but has gained more interest recently [Roggendorf *et al.*, 1996]. Early studies revealed a greater presence of macrophages and microglia in GBM compared to lower grade malignancies [Roggendorf *et al.*, 1996]. Microglia are a

subtype of macrophages found specifically in the brain and spinal cord that form an important part of the glial or non-neuronal supporting cells within the central nervous system. The source of both macrophages and microglia present in GBM is thought to be bone marrow-derived monocytes that can migrate to the brain during adulthood [Guillemin and Brew, 2004]. The macrophages and microglia can be recruited to the tumor site through growth factors and cytokines produced by the tumor cells. Cytokines such as monocyte chemoattractant protein-1 (MCP-1) and SDF-1 produced by GBM cells may serve to attract macrophages and microglia to the tumor [Watters *et al.*, 2005]. Distinctions between macrophages and microglia are difficult to define as microglia express many of the same cell surface markers as macrophages such as CD11b/c and CD45 [Watters *et al.*, 2005]. As such, this document will focus on the combined contributions of both cell types to GBM and refer to them collectively as macrophages/microglia.

Macrophages/microglia present in GBM are thought to contribute to a variety of pro-tumorigenic processes including immunosuppression, angiogenesis, GBM proliferation and migration. A study of freshly isolated GBM tumors and six human glioma cell lines found high expression of immunosuppressive cytokines and their receptors such as leukemia inhibitory factor (LIF) which is involved in differentiation, oncostatin-M (OSM) which can inhibit the production of pro-inflammatory cytokine TNF $\alpha$ , and TGF- $\beta$  which can block the activation of lymphocytes and macrophages [Hao *et al.*, 2002; Watters *et al.*, 2005]. Macrophages/microglia are likely the main source of the immunosuppressive cytokine IL-10 that can promote glioma cell

proliferation [Watters *et al.*, 2005]. These factors can also affect GBM migration, contributing to the invasive and infiltrative nature of GBM. VEGF, an important factor in angiogenesis mentioned above, is expressed by both macrophages/microglia and GBM cells. Also, the presence of VEGF can support GBM survival and promote macrophage/microglial proliferation whose presence further supports the tumor's survival, creating a positive feedback loop [Watters *et al.*, 2005]. Another important factor produced by macrophages/microglia is TNF $\alpha$  which can induce the migration of leukocytes into GBM and induce expression of factors that support tumor growth including VEGF (angiogenesis), EGFR (proliferation), and MMP-9 (migration) [Watters *et al.*, 2005]. In GBM and other tumor types, there is a desire to target macrophages/microglia, either to inhibit their tumor-promoting interactions with GBM cells or activate the pro-inflammatory response of macrophages/microglia against GBM cells. The collective contributions of GBM cells, macrophage/microglia and other cells of the tumor microenvironment drive the hallmark features of GBM. Further research is needed to devise novel therapeutics that target pathways known to be involved in GBM tumorigenesis and identify more pathways that contribute to tumor progression through these myeloid cells.

#### **1.1.2.2 Pancreatic Cancer**

Pancreatic ductal adenocarcinoma (PDA), referred to as pancreatic cancer in this document, is the fourth leading cause of cancer-related deaths in the United States with a very poor prognosis. In 2010, 43,140 people were diagnosed with

pancreatic cancer while 36,800 people died from the disease, reflecting the poor prognosis of this type of cancer [Jemal *et al.*, 2010]. The median survival rate is six months while the overall 5-year survival rate is less than 5% [Korc, 2007; Warshaw and Fernandez-del Castillo, 1992]. These statistics have remained mostly unchanged for over a decade, partly because pancreatic cancer is usually only apparent in late stages when it is more difficult to treat, and because the disease is often resistant to conventional chemotherapy and radiation therapy [Hingorani *et al.*, 2003]. Factors that can hinder earlier diagnosis include the retroperitoneal location of the pancreas and the small precursor lesions that are undetectable with current imaging methods. Even when detected at an early stage and resected, many patients succumb to the disease due to recurrence or metastasis [Korc, 2007].

The standard of care treatment for pancreatic cancer is gemcitabine (GEM) after a clinical trial demonstrated GEM's ability to increase the median disease-free survival and overall survival of patients to a greater extent than fluorouracil (5-FU) [Burris *et al.*, 1997]. GEM (difluorodeoxycytidine; dFdC) is a nucleoside analog that, when phosphorylated intracellularly to yield dFdCTP, competes with deoxycytidine triphosphate (dCTP) for incorporation into DNA, inhibiting DNA synthesis and blocking tumor cell proliferation [Heinemann *et al.*, 1988; Huang *et al.*, 1991]. While extensive trials with GEM have been conducted to improve efficacy, little improvement has been made in survival outcomes [Chames *et al.*, 2010]. There is a small clinical benefit to combining GEM with erlotinib, an anti-EGFR tyrosine kinase inhibitor, or platinum, however, increased toxicity is experienced and only the

GEM/erlotinib combination is FDA approved, improving median overall survival from 6 months to 6.4 months [Moore *et al.*, 2007; Senderowicz *et al.*, 2007]. While this improvement in survival is not extensive, it is largely accepted that combination therapy will be the best way to combat this complex disease.

Identifying and distinguishing major variant types of pancreatic cancer has helped diagnoses and therapeutic strategies in recent years [Kern *et al.*, 2010]. The completion of the Pancreatic Cancer Genome Project will provide a database of mutations and copy number variations that can be used to set boundaries on the frequencies of individual variation [Jones *et al.*, 2008]. Certain genes are known to have a high frequency of mutations in pancreatic cancer including *Kras*, *p53*, *Smad4* and *p16* which can enhance tumor cell proliferation, suppress apoptotic pathways and promote tumor metastasis [Hansel *et al.*, 2003]. However, stromal elements of the pancreatic cancer microenvironment also contribute to tumor progression [Korc, 2007]. These stromal elements include proliferating fibroblasts and pancreatic stellate cells that produce and deposit fibronectin and collagens I and III, contributing to the desmoplastic nature of the stroma, as well as endothelial cells, pericytes and macrophages which produce chemokines and cytokines that can direct functions of many of the cells in the tumor microenvironment [Korc, 2007].

Targeting signaling pathways important for these cell types such as EGF, fibroblast growth factor (FGF), PDGF, insulin-like growth factor (IGF), TGF- $\beta$  and VEGF may disrupt the stromal-epithelial interactions that drive tumor growth. Unfortunately, recent clinical trials have not yielded encouraging results with stromal-

directed therapeutics. As mentioned above, combination treatment of GEM and erlotinib offered slightly improved survival with increased toxicity as a side effect. Two recent phase II clinical trials combined anti-VEGF receptor drug bevacizumab with GEM and saw either no benefit or a slight benefit with significant toxicity, and both concluded the regimens should not be further pursued [Fogelman *et al.*, 2011; Ko *et al.*, 2011]. Because of these setbacks, further research is needed to identify targetable components of the microenvironment of pancreatic cancer and improve inhibitors.

One cell type in the microenvironment of pancreatic cancer that has been singled out as a contributor to tumor progression is a subset of myeloid cells, TAMs. TAMs are derived from bone marrow progenitors that develop into monocytes, extravasate and infiltrate into the tumor tissue where they differentiate into macrophages [Kurahara *et al.*, 2011]. Among macrophages, there are subpopulations that are defined by the cytokines that activate them, as mentioned previously. Some macrophages are activated by an alternative pathway involving T helper 2 (Th2) cytokines such as IL-4 and IL-13, and are referred to as M2-polarized macrophages [Kurahara *et al.*, 2011]. M2 macrophages promote angiogenesis and tissue remodeling in contrast to M1 macrophages which kill microorganisms and tumor cells. TAMs have been identified as M2-polarized macrophages and receive cues from the tumor microenvironment to promote tumor cell invasion, induce angiogenesis and suppress antitumor immunity, as well as aiding in metastasis [Clark *et al.*, 2007; Pollard, 2004]. A study comparing M1 and M2-polarized

macrophage infiltration in pancreatic cancers found that M2-polarized TAMs were present in the invasive front of pancreatic cancer and could promote lymphangiogenesis, lymphatic metastasis and could be a predictor for poor prognosis [Kurahara *et al.*, 2011]. Therapies aimed at disrupting the interactions between tumor cells and stromal cells such as TAMs are a promising area of research.

## **1.2 Targeting the Tumor Microenvironment**

As we gain understanding about the tumor cells and their microenvironment, there is great interest in targeting the supporting cells of the tumor microenvironment in addition to the tumor cells as a way to combat the disease. There are different subpopulations of cells that are being targeted in current research, such as endothelial and immune cells. Endothelial cells are of particular interest because of their involvement in angiogenesis, a process that supplies tumors with the oxygen and nutrients they need to survive. However, as discussed below, eradicating endothelial cells and inhibiting angiogenesis is not the silver bullet some thought it would be; rather, it often results in resistance and refractoriness in some cancer types. We are gaining more knowledge about the cells of the immune system that infiltrate tumors to promote tumor progression and there is interest in studying how to target these immune cells to inhibit tumor growth.

### **1.2.1 Anti-Angiogenic Therapies and Their Limitations**

With the cloning of VEGF in 1989 came a further understanding of tumor angiogenesis and VEGF's central role in the process [Keck *et al.*, 1989; Leung *et al.*, 1989]. The VEGF family of growth factors consists of 5 structurally similar members: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGF) [Kerbel, 2008]. VEGF-A is the major mediator in angiogenesis and is usually referred to as VEGF. Interactions between VEGF and the receptor tyrosine kinase (RTK) VEGF receptor 2 (VEGFR-2) contribute most significantly to angiogenesis through downstream signals. These downstream signals include activation of the MAPK pathway which leads to initiation of DNA synthesis and cell proliferation while activation of the PI3K/Akt pathway can promote endothelial cell survival [Kerbel, 2008]. The extensive angiogenic effects of the VEGF signaling pathway made it an ideal target for anti-cancer therapeutics.

Less than two decades after the discovery of the VEGF signaling pathway, the first VEGF-targeted therapy, the monoclonal antibody bevacizumab (Avastin, Genentech) was introduced and shown to have beneficial effects on patients with colorectal cancer (CRC) when combined with chemotherapy [Hurwitz *et al.*, 2004]. In 2004, bevacizumab was approved by the Food and Drug Administration (FDA) for treatment of CRC and non-small cell lung cancer (NSCLC). Since that time, other anti-VEGF therapies have been developed that target the VEGF receptor (VEGFR),

such as sorafenib (Nexavar, Bayer/Onyx) and sunitinib (Sutent, Pfizer) [Ellis and Hicklin, 2008].

Currently, bevacizumab is approved for treating metastatic CRC, NSCLC and metastatic breast cancer [Hurwitz *et al.*, 2004; Miller *et al.*, 2007; Sandler *et al.*, 2006]. Sorafenib has shown efficacy in advanced renal cell carcinoma (RCC) and hepatocellular carcinoma (HCC), while sunitinib has also shown efficacy in RCC [Escudier *et al.*, 2007; Llovet *et al.*, 2008; Motzer *et al.*, 2007]. The mechanism of action of anti-VEGF therapies may vary by tumor type, and there are a variety of ways that anti-VEGF therapies may inhibit tumor growth.

The anti-angiogenic effects of anti-VEGF therapy may include the inhibition of new blood vessel formation, induction of endothelial cell apoptosis and blocking the incorporation of hematopoietic and endothelial progenitor cells [Ellis and Hicklin, 2008]. Anti-VEGF therapy can also affect blood vessel function, causing vessel constriction and normalization, both of which can better regulate blood flow to the tumor and improve delivery of chemotherapy and oxygen to the tumor [Ellis and Hicklin, 2008]. While the results with anti-VEGF therapies were initially encouraging, some cancers treated with these therapies became resistant or refractory to the treatment while others have no response [Fogelman *et al.*, 2011; Ko *et al.*, 2011; Norden *et al.*, 2008].

The drawbacks and limitation of anti-VEGF therapy are also important to understand to further comprehend how anti-angiogenic therapies can be improved. Two initial drawbacks of some anti-VEGF therapies include high cost and toxicity

associated with other monoclonal antibody therapies [Kerbel, 2008]. Some recognized patterns of toxicity for anti-VEGF therapies include hypertension, bleeding and perforations [Eskens and Verweij, 2006]. While treatment with anti-VEGF therapies may be short term, and, therefore, the effects of toxicity may be manageable, long term use may be prevented by increased toxicity [Verheul and Pinedo, 2007]. Another concern with anti-VEGF treatment in the clinic is the observation of intrinsic and acquired resistance of tumors to anti-angiogenic drugs.

Resistance or refractoriness to anti-angiogenic treatment, specifically, anti-VEGF therapies, is an important concern in the clinical setting. Intrinsic, or pre-existing, resistance is observed in tumors that are completely non-responsive to anti-VEGF therapy [Bergers and Hanahan, 2008; Kerbel, 2008]. In clinical trials, this intrinsic resistance is demonstrated in patients that failed to show even temporary benefits after treatments with bevacizumab, sorafenib or sunitinib [Bergers and Hanahan, 2008]. Recent studies have shown that some cell lines are intrinsically resistant to anti-VEGF treatment as alternative mechanisms can contribute to tumor growth even after treatment with anti-VEGF therapy. In one study, CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cell infiltration was shown to be associated with anti-VEGF refractoriness in models of lymphoma (EL4 cells) and lung cancer (Lewis lung carcinoma or LLC cells) as anti-Gr-1 antibodies combined with anti-VEGF treatment inhibited the refractory growth of tumors more effectively than anti-VEGF alone [Shojaei *et al.*, 2007a]. Intrinsic resistance can also occur when VEGF or VEGF receptors are not

present, an occurrence sometimes seen in metastatic tumors [Karashima *et al.*, 2007].

Acquired or evasive resistance to anti-VEGF therapy represents an adaptation to evade the angiogenic blockade and can occur when other angiogenic factors are upregulated in response to inhibition of the VEGF pathway, exploiting the redundancy of angiogenesis stimulators [Bergers and Hanahan, 2008; Kerbel, 2008]. Some of these factors include bFGF, G-CSF, SDF-1, angiopoietins and ephrins [Bergers and Hanahan, 2008; Kerbel, 2008].

A number of studies have examined the effects of anti-VEGF therapy in models of GBM and pancreatic cancer or patients and found many of the tumors to be resistant or have a refractory response. In an orthotopic model of GBM, mice receiving anti-VEGF antibody treatment showed prolonged survival compared to control treatment, but increased infiltration of normal brain tissue by tumor cells and cooption of the host vasculature was observed, leading to the conclusion that anti-angiogenic strategies alone would not be successful in GBM [Rubenstein *et al.*, 2000]. Another study found correlations between patients with GBM treated with bevacizumab and mice with orthotopic xenograft GBM treated with bevacizumab using both radiographic imaging (magnetic resonance imaging or MRI) and immunohistochemistry (IHC). The study found an increase in progression-free survival but not overall survival duration in patients, and GBM tumors in both patients and mice exhibited increased infiltrative patterns characterized by tumor cells infiltrating the surrounding normal brain tissue [de Groot *et al.*, 2010]. Researchers

concluded the increase in invasiveness was in response to the bevacizumab treatment that can increase hypoxia and decrease nutrient supply. In a clinical study, combination treatment of chemotherapy and bevacizumab in patients with GBM showed little long-term disease control with lesser effects observed in suppressing infiltrative tumor growth into normal brain tissue [Norden *et al.*, 2008]. The researchers concluded that the bevcizumab treatment may decrease suppression of infiltrative tumor growth, or the treatment may have significantly reduced the tumor capillary permeability, decreasing the delivery of chemotherapeutic treatment. Treatment with sunitinib and VEGFR-selective kinase inhibitors in mice with orthotopic injections of transformed mouse astrocytes also resulted in an increased invasiveness phenotype, and there was no observed survival advantage [Paez-Ribes *et al.*, 2009]. Resistance or refractoriness has also been observed in pancreatic cancer.

Multiple studies with models of pancreatic cancer and clinic trials in pancreatic cancer have observed resistance or refractory responses to varying anti-VEGF therapeutics. In a mouse model of pancreatic islet carcinogenesis (*RIP1-Tag2,Rag<sup>-/-</sup>*), resistance to VEGFR2 inhibition was observed as tumors regrew after initial growth suppression, mediated by VEGF-independent angiogenesis contributed to by proangiogenic factors upregulated by hypoxia including FGF family members [Casanovas *et al.*, 2005]. In another study of a model of pancreatic cancer, tumors of rat insulin promoter-T antigen (RIP-Tag) mice treated with an anti-VEGFR2 antibody showed increased invasiveness and metastasis, and similar results were also seen

after treatment with sunitinib [Paez-Ribes *et al.*, 2009]. Two clinical trials combining bevacizumab with other therapeutics to treat pancreatic cancer found significant toxicity without significant prolonged survival [Carbone *et al.*, 2011; Ko *et al.*, 2011]. Therefore, treatment with anti-VEGF therapeutics may not represent the most effective treatment options for GBM and pancreatic cancer. Further evidence suggests that anti-VEGF treatment can promote pro-tumorigenic processes in cancer, including recruitment of myeloid cells.

Anti-VEGF treatment can also lead to recruitment of bone marrow-derived cells (BMDCs) that can reconstitute the vasculature in a variety of ways. Pro-angiogenic BMDCs can consist of vascular progenitors such as endothelial and pericyte progenitors that can differentiate into endothelial cells and pericytes to form blood vessel structures [Bergers and Hanahan, 2008]. Alternatively, these BMDCs may consist of pro-angiogenic CD11b<sup>+</sup> myeloid cells such as immature monocytes, monocytes and TAMs. These cell types can exert pro-angiogenic functions through expression of cytokines, growth factors and proteases without being physically part of the vessel structures [Bergers and Hanahan, 2008]. Indeed, a study of GBM found that hypoxia induced HIF-1 $\alpha$  promoted angiogenesis through recruitment of BMDCs, including myeloid cells and macrophages [Du *et al.*, 2008]. GBM tumors that lacked HIF-1 $\alpha$  showed fewer BMDCs, and their angiogenic and tumor growth phenotypes were impaired. Research is now focused on the role of these myeloid cells in angiogenesis and other pro-tumorigenic aspects of cancer. There is a desire to develop drugs to target these cells to treat cancer.

## 1.2.2 Targeting Alternative Pathways of the Tumor Microenvironment

Because some cancers like GBM and pancreatic cancer exhibit resistance or refractoriness to anti-VEGF therapeutics, there is a need to identify and study alternative signaling pathways in the tumor microenvironment that contribute to tumorigenesis. Myeloid cells of the immune system that are present in the tumor microenvironment have been widely studied and are important contributors to tumor progression. There are several potential approaches to targeting the myeloid cell population of the stromal compartment of the tumor microenvironment that supports cancer progression. These methods include eliminating the myeloid cells, promoting differentiation of myeloid progenitors, and inhibiting the function of the infiltrating myeloid cells by targeting inflammatory cytokines [Kusmartsev and Gabrilovich, 2006; Pollard, 2004].

Tumor growth suppression can be mediated by decreased CTL activity where, in normal immune settings, CD8<sup>+</sup> CTLs are generated from activated and differentiated naïve T cells and these CTLs then destroy antigen-presenting target cells (APCs) [Solito *et al.*, 2011; Weninger *et al.*, 2002]. CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells can disrupt this immune response by suppressing T cell function through cell-surface interactions with surface molecules like CD80, CD115 and CD124 and the release of myeloid-activating cytokines such as INF $\gamma$ , IL-4, IL-13 and TGF- $\beta$  [Gabrilovich and Nagaraj, 2009; Solito *et al.*, 2011]. Two studies have shown that depleting Gr1<sup>+</sup> cells can enhance tumor growth suppression. In one study, depletion of Gr1<sup>+</sup> cells through

anti-Gr1 antibody treatment resulted in improved CD8<sup>+</sup> T cell immune response in spontaneous UV light-induced tumors in mice, eradicating the tumors [Seung *et al.*, 1995]. Another study identified Gr1<sup>+</sup> cells as the main source of TGF- $\beta$  that promotes tumor growth through downregulating CTL-mediated tumor immunosurveillance [Terabe *et al.*, 2003]. Neutralizing antibodies against Gr1 and TGF- $\beta$  prevented fibrosarcoma tumor recurrence in mice. Therefore, there is interest in CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells, how they are regulated and how they affect tumor progression including the cytokines they produce and the signaling pathways to which they contribute.

#### **1.2.2.1 PK2, a Regulatory Peptide and Member of the Prokineticin Family**

Among those factors produced by the CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells is prokineticin 2 or PK2, also known as Bv8. An ortholog of PK2 named Bv8 was originally identified as a novel 8 kDa peptide isolated from the skin of the frog species *Bombina variegata* and identified as a homologue of MIT1 (protein A) from black mamba (*Dendroaspis polylepis*) snake venom [Mollay *et al.*, 1999]. Initial studies showed the ability of both peptides to stimulate contraction of gastrointestinal (GI) smooth muscle [Mollay *et al.*, 1999; Schweitz *et al.*, 1990; Schweitz *et al.*, 1999]. Mammalian homologues of Bv8 and MIT1 were later identified and named prokineticin 2 (PK2) and prokineticin 1 (PK1), respectively [LeCouter *et al.*, 2001; Li *et al.*, 2001; Wechselberger *et al.*, 1999]. PK1 and PK2 share approximately 45% amino acid identity with each other and with *B. variegata* Bv8 and *D. polylepis* MIT1

[Li *et al.*, 2001]. PK2 and PK1 each bind to two highly related G-protein-coupled receptors (GPCRs), prokineticin receptor 1 (PKR1) and prokineticin receptor 2 (PKR2), with similar agonist potency [Lin *et al.*, 2002]. Briefly, GPCRs are seven pass transmembrane protein receptors that recognize and transduce messages from diverse signals such as light, calcium ( $\text{Ca}^{2+}$ ), nucleotides, amino acids, peptides and proteins [Bockaert and Pin, 1999]. These signals through the GPCR can control the activity of enzymes, ion channels and the transport of vesicles, mediated by the exchange of GDP for GTP on the heterotrimeric G proteins ( $\text{G}\alpha\text{-}\beta\gamma$ ) [Bockaert and Pin, 1999]. The GTP-bound  $\text{G}\alpha$  subunit affects further downstream signals depending on its subtype. The binding of PK2 to PKR1 or PKR2 stimulates  $\text{Ca}^{2+}$  mobilization from its intracellular stores, phosphoinositol turnover and activation of Akt kinase and MAPK through  $\text{G}\alpha_{\text{i}}$  and  $\text{G}\alpha_{\text{q}}$  subtype signaling [Lin *et al.*, 2002; Zhou, 2006]. The activation of PKR1 and PKR2 can affect multiple biological processes including nociception, circadian rhythm, GI motility, neurogenesis, hematopoiesis and angiogenesis [Zhou, 2006]. The effects of PK2 may be determined by the expression patterns of PKR1 and PKR2 as PKR1 is widely expressed in the periphery including the GI system, lungs and blood system, while PKR2 is expressed in the adult brain. Both receptors are expressed in several endocrine tissues including thyroid, pituitary, adrenal gland, testis and ovary [Zhou, 2006].

Initial functional studies of the PKs focused on their role in GI smooth muscle cell contractility, but with their extensive expression pattern, they have been implicated in numerous physiological processes. PK signaling has been implicated in

nociception with excess PK2 causing strong and localized hyperalgesia in rats [Mollay *et al.*, 1999]. One of the most extensive areas of research for PK2 has been in the suprachiasmatic nucleus (SCN) in the anterior hypothalamus of the mouse brain where levels of PK2 mRNA display circadian rhythmicity under light/dark cycling and constant dark suggesting that PK2 is involved in the circadian clock in mammals [Cheng *et al.*, 2002]. Expression of PK2 in this manner is controlled by E-boxes under the regulation of the CLOCK-BMAL1 transactivation complex [Cheng *et al.*, 2002]. PK2-null mice display reduced circadian locomotor rhythmicity and reduced rhythmicity for the sleep/wake cycle, body temperature, circulating glucocorticoid and glucose levels, and expression of peripheral clock genes [Zhou, 2006].

Recently, the role of PK2 in angiogenesis and the development and function of hematopoietic cells has been extensively studied and described. PK2 induces proliferation, survival and migration of adrenal cortical capillary endothelial (ACE) cells, and its expression can be induced by hypoxia [LeCouter *et al.*, 2003]. Expression of PK2 and its receptors in bone marrow has led to research into their role in hematopoiesis. Indeed, PK2 expression supports the differentiation of the monocytic lineage and promotes the survival and differentiation of granulocytic lineages [LeCouter *et al.*, 2004]. Further, PK2 is highly expressed by leukocytes infiltrating sites of inflammation, and PK2 stimulates primary monocyte migration [LeCouter *et al.*, 2004]. The link between PK2 expression in myeloid cells such as neutrophils and monocytes/macrophages and myeloid cell infiltration, processes

important for cancer progression, have produced compelling evidence that PK2 signaling is involved in creating a favorable tumor microenvironment for growth of tumors.

#### 1.2.2.2 PK2 Regulates Myeloid-Cell Dependent Tumor Progression

PK2 production by CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells can lead to the formation of a positive feedback loop as tumor cells can promote myeloid cell differentiation and mobilization from the bone marrow into the blood stream and tumor sites and PK2 production by myeloid cells can promote tumor growth [Shojaei *et al.*, 2007b]. These differentiated macrophages can infiltrate the tumor microenvironment and continue to secrete more PK2, leading to increased proliferation of endothelial cells expressing PKR1 and PKR2, thus contributing to enhanced angiogenesis [Shojaei *et al.*, 2007b]. In addition to targeting endothelial cells, PK2 was shown to affect cytokine production in mouse lymphocytes, increasing pro-inflammatory cytokines IL-1 and IL-12 while decreasing anti-inflammatory cytokines IL-4 and IL-10 [Franchi *et al.*, 2008; Martucci *et al.*, 2006]. PK2 and its receptors PKR2 and, to a greater extent PKR1, are also expressed by mouse macrophages; consequently PK2 can induce migration of these macrophages [Martucci *et al.*, 2006].

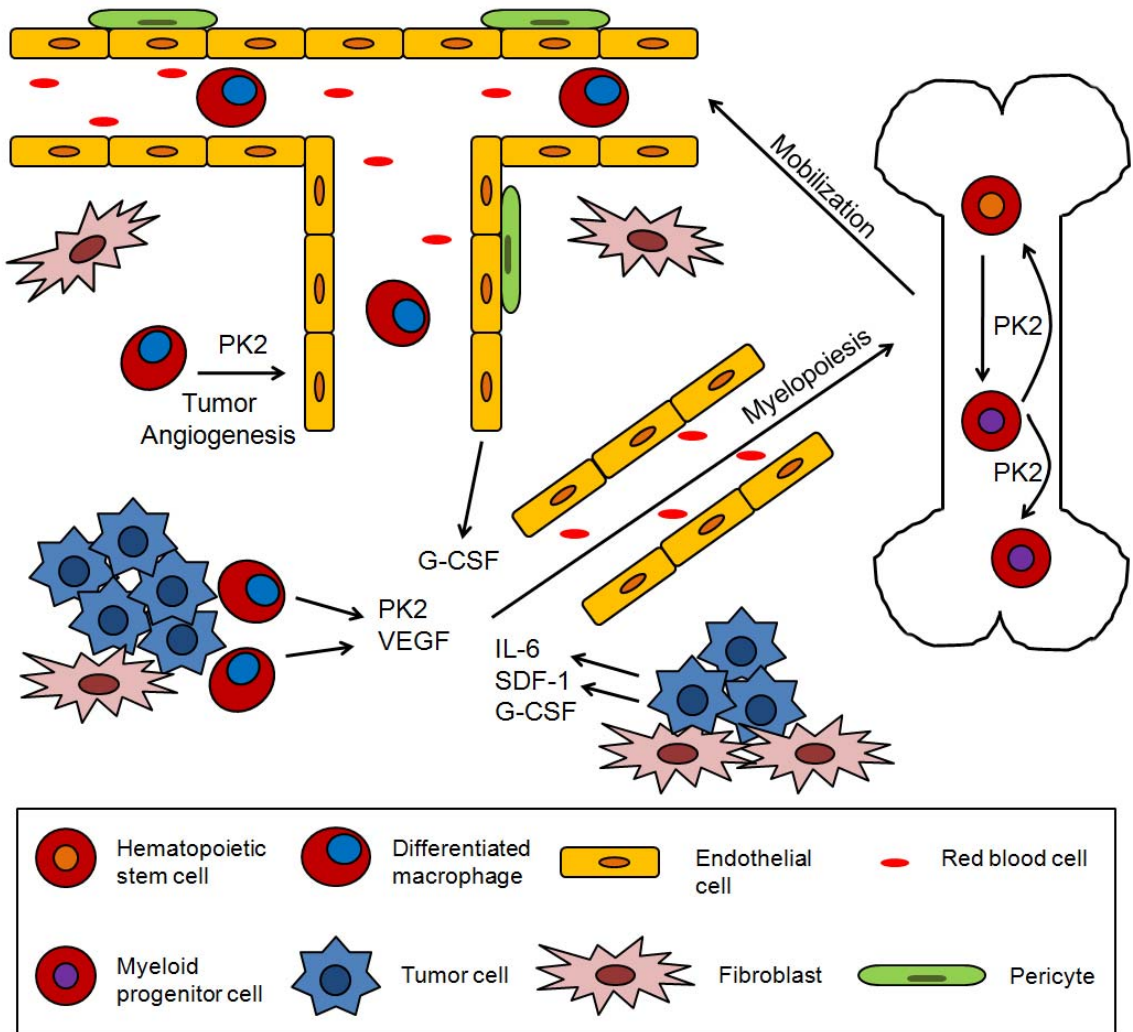
Because of the important roles played by PK2 in the creation of a tumor microenvironment favoring growth and progression, PK2 has become a target for the development of novel cancer therapies. A number of studies have convincingly shown that neutralizing antibodies against PK2 can exhibit a potent anti-tumor effect

on multiple types of human cancers in mouse xenograft models. PK2 expression in bone marrow can be upregulated by G-CSF, a protein constitutively expressed by tumor cells [Mueller and Fusenig, 2002]. This upregulation of PK2 in CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells by G-CSF also contributes to the mobilization of these myeloid cells from the bone marrow into the bloodstream and into the tumor [Shojaei *et al.*, 2007b]. Anti-PK2 antibody treatment reduced CD11b<sup>+</sup>Gr1<sup>+</sup> cell mobilization from the bone marrow, as fewer CD11b<sup>+</sup>Gr1<sup>+</sup> cells were measured in the peripheral blood of mice treated with anti-PK2 antibody [Shojaei *et al.*, 2007b]. Anti-PK2 antibodies also inhibited xenograft growth of pancreatic cancer (HPAC), colon cancer (HM7), rhabdomyosarcoma (A673) and lymphoblast (Jurkat) tumors in mice and suppressed angiogenesis while reducing CD11b<sup>+</sup>Gr1<sup>+</sup> cells in peripheral blood and tumors, further illustrating the importance of myeloid cells in tumorigenesis [Shojaei *et al.*, 2007b].

Anti-PK2 antibody treatment was further tested in the RIP-Tag transgenic mouse model of pancreatic  $\beta$ -cell tumorigenesis by Shojaei *et al.* When compared to treatment with an anti-VEGF antibody, the anti-PK2 treatment was as effective in preventing disease progression as the anti-VEGF treatment, reducing angiogenic islets and mobilization and homing of CD11b<sup>+</sup>Gr1<sup>+</sup> cells to peripheral blood and neoplastic lesions [Shojaei *et al.*, 2008a]. There was no effect on tumor vascularization or growth when anti-PK2 treatment was initiated at later stages of disease progression, suggesting that PK2 was an important mediator of CD11b<sup>+</sup>Gr1<sup>+</sup>

myeloid cell-dependent angiogenesis, critical for early stages of tumor progression [Shojaei *et al.*, 2008a].

Refractoriness to anti-VEGF treatment is mediated by CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells in certain tumor cell types, specifically mouse lymphoma EL4 cells and LLC cells [Shojaei *et al.*, 2007a]. A follow up study revealed that G-CSF and PK2 were preferentially expressed in these refractory EL4 and LLC tumors suggesting the PK pathway may mediate anti-VEGF refractoriness [Shojaei *et al.*, 2009]. Combining anti-G-CSF or anti-PK2 with anti-VEGF treatment delayed subcutaneous (SC) EL4 and LLC tumor growth [Shojaei *et al.*, 2009]. These combination therapy regimens also inhibited tumor angiogenesis as the vascular surface area, measured by CD31 staining, was significantly reduced in tumors receiving both treatments compared to those receiving only anti-VEGF treatment [Shojaei *et al.*, 2009]. This finding further validates an anti-angiogenic mechanism for anti-PK2 antibody treatment. A more recent study with anti-PK2 antibodies demonstrated that anti-G-CSF and anti-PK2 treatment can reduce lung metastasis of mouse mammary cells lines (66c14, 4T1 and MMTV-PyMT) and a human breast cancer cell line (MDA-MB-231) after implantation into the mammary fat pad of mice [Kowanetz *et al.*, 2010]. These studies have led to a model of how PK2 can contribute to tumor progression and angiogenesis through myeloid cells, (Figure 1.1) [Shojaei and Ferrara, 2008a].



**Figure 1.1 Role of myeloid cells and PK2 in tumor growth through angiogenesis**

The secretion of cytokines such as IL-6, SDF-1 and G-CSF from tumor cells leads to myelopoiesis in the bone marrow where hematopoietic stem cells differentiate into myeloid progenitor cells. These myeloid progenitor cells secrete PK2, further expanding their population. These cells then mobilize from the bone, differentiate into macrophages and travel through the circulation to tumor sites. Further secretion of PK2 can lead to angiogenesis and a positive feedback loop, recruiting more tumor-promoting macrophages.

Therefore, anti-PK2 treatments may have many applications for different cancer types, and further research is needed to explore therapeutic options. As stated above, there are certain disadvantages to antibody-based therapeutics like bevacizumab such as high costs [Kerbel, 2008]. Small molecule inhibitors offer specific advantages over antibodies as therapeutic agents. Both small molecules and antibodies can be specific to their targets but small molecules have lower manufacturing costs and are smaller than antibodies, enabling small molecules to cross the blood-brain barrier (BBB), a feature important in treating GBM [Imai and Takaoka, 2006].

### ***1.3 Summary and Hypothesis***

Many different cell types present in the tumor microenvironment can play a role in cancer progression. In certain cancer types, such as GBM and pancreatic cancer, immune cells like CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells present in the tumor microenvironment can contribute to tumor progression in a variety of ways. The myeloid cells can enhance angiogenesis, supplying the tumor with oxygen and nutrients, or express pro-tumorigenic cytokines, suppressing the immune response and supporting aggressive tumor growth. While therapeutics have been developed to target certain cells and processes of the tumor microenvironment, many of these treatments have resulted in resistance or refractoriness, especially in GBM and pancreatic cancer. Other signaling pathways that are involved in pro-tumorigenic roles in the tumor microenvironment, including the role of myeloid cells, have been

actively pursued by researchers. The prokineticin signaling pathway has been identified as important for myeloid cell maturation and mobilization as well as their function in supporting tumor growth.

Both GBM and pancreatic cancer have significant pro-tumorigenic contributions from myeloid cells mediated by PK2. We believe a small molecule inhibitor against the PK2 signaling pathway targeting myeloid cell function in models of GBM and pancreatic cancer could offer a viable therapeutic option for these cancer types superior to previous antibody-based therapies. The following chapters detail our results from our experiments, conclusions based on our observations and future directions we hope to pursue in further developing PK signaling inhibitors as a therapeutic treatment.

## **2. Materials and Methods**

### **2.1 Cell Culture**

#### **2.1.1 D456MG, T4105**

D456MG and T4105 cells were cultured in Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented and maintained as described [Wang *et al.*, 2009].

#### **2.1.2 AsPc-1, CFPac-1, THP-1, RAW264.7**

AsPc-1, CFPac-1, THP-1 and RAW264.7 cells were cultured in RPMI 1640 medium (Mediatech, Inc, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and Penicillin/Streptomycin Solution (P/S; Mediatech, Inc).

#### **2.1.3 IHMVEC, MEEC, HMEC-1**

IHMVEC were cultured in EBM-2 (Lonza, Basel, Switzerland), EGM-2 MV SingleQuots (Lonza), P/S.

MEEC and HMEC-1 were cultured in MCDB-131 (Invitrogen). MEEC medium was supplemented with 15% FBS, 2mM L-glutamine, 1mM sodium pyruvate (Invitrogen), 100µg/mL of heparin (Sigma-Aldrich, St. Louis, MO), and 50µg/ml endothelial cell growth supplement (ECGS) (Sigma-Aldrich). HMEC-1 was supplemented with 10% FBS, 1µg/ml hydrocortisone (Sigma), 10ng/ml EGF (Sigma) and 2mM L-glutamine (Invitrogen).

## **2.2 Tumor Xenografts**

### **2.2.1 Subcutaneous Tumor Xenografts**

Athymic *nu/nu* (nude) mice were maintained in HEPA-filtered facilities in the Duke University Cancer Center Isolation Facility. SC transplantations of D456MG, AsPc-1 and CFPac-1 cells into these mice were performed as described [Li *et al.*, 2009; Rich *et al.*, 2005]. For SC injections,  $5 \times 10^4$  D456MG,  $5 \times 10^5$  AsPc-1 or  $5 \times 10^5$  CFPac-1 cells were implanted subcutaneously on the right flank of nude mice in a volume of 50 $\mu$ l using a 28G1/2" insulin syringe (Becton Dickinson, Franklin Lakes, NJ). SC tumors were measured with hand-held vernier calipers (Bel-Art Products, Pequannock, NJ) and tumor volume was calculated based on the following formula:  $[(\pi/6) \times (\text{width})^2 \times (\text{length})]$ . In all animal experiments, animals were treated with 20mg/kg PKRA7 or phosphate-buffered saline injected intraperitoneally (IP) with previously mentioned insulin syringes every day until the termination of experiments, at which time mice were sacrificed and tumors were harvested, weighed, and examined. In combination experiments, as well as daily injections of 20mg/kg PKRA7, 100mg/kg GEM (Eli Lilly, Indianapolis, IN) was administered to AsPc-1 SC injected mice 1 week after injection and received 4 total injections for 2 weeks (4 days apart).

### **2.2.2 Intracranial Tumor Xenograft Injections**

Athymic *nu/nu* mice were maintained in HEPA-filtered facilities in the Duke University Cancer Center Isolation Facility. Intracranial (IC) transplantations of D456MG and T4105 cells into these mice were performed as described [Li *et al.*, 2009; Rich *et al.*, 2005]. Briefly, for IC transplantations,  $1 \times 10^4$  D456MG or T4105 cells were implanted into the subventricular zone of the brains of 4-6 week old mice using previously mentioned syringes. Mice were maintained until the development of neurological symptoms. In all animal experiments, animals were treated with 20mg/kg PKRA7 or phosphate-buffered saline (PBS) injected IP with previously mentioned insulin syringes every day until the termination of experiments, at which time mice were sacrificed and brains were harvested. In combination experiments, as well as daily injections of 20mg/kg PKRA7, 10mg/kg of TMZ was administered to mice receiving D456MG IC injections 3 days after injection and received 5 consecutive treatments.

### **2.2.3 Immunohistochemistry and Analysis**

Tumors collected from sacrificed mice were embedded in paraffin and stained with H&E or desired antibodies for IHC analysis and counterstained with hematoxylin.

For necrotic areas, 5 slides per tumor (both D456MG and AsPc-1) were H&E stained. High resolution images were taken of each slide at 1x magnification and these slides were analyzed using the ImageJ program. For each slide, the area of

the total tumor section was measured in ImageJ. Then, areas identified histologically as necrotic were also measured in ImageJ. The ratio of necrotic area per tumor section over the total tumor section was calculated for each tumor section. These calculations were averaged in two groups: mice receiving control treatment and those receiving PKRA7 treatment.

For measuring CD34 positive cells, 5 slides per tumor D456MG and 1 slide per AsPc-1 tumor were stained by mouse CD34 antibody (Abcam, Cambridge, UK). For D456MG tumor sections, five images were taken at 20x of each slide (total 25 fields for each tumor) and these images were analyzed using the ImageJ program. For each field, the area of CD34-positive shown in brown was measured in ImageJ. Then, the ratios of CD34 positive area compared to the area of whole field were calculated and analyzed. For AsPc-1 tumors, 5 fields at 10X per tumor section were analyzed and CD34-positive vessels were counted and averaged per field of view.

For measuring F4/80 positive cells, 1 slide per AsPc-1 subcutaneous tumor and 3 slides of 2 brains per condition from IC D456MG injections were stained by the murine macrophage marker, F4/80 antibody (AbD Serotec, Oxford, UK). For the AsPc-1 tumors, high resolution images were taken at 10x, with 3 to 4 images taken per tumor section, depending on its size. The number of positively stained cells was counted on each image. These numbers were averaged as macrophage count per field for mice receiving control treatment and those receiving PKRA7 treatment. For brain sections containing D456MG tumors, the number of macrophages per 10x field was counted and averaged.

## **2.3 In Vitro Angiogenesis Assay**

### **2.3.1 Capillary Branching Assay**

Twelve-well plates were coated with 200µl Matrigel Matrix (BD Biosciences, San Jose, CA) and allowed to solidify at 37°C. For IHMVEC cells,  $3 \times 10^4$  cells were plated in 1ml EBM-2 media with the following treatment conditions: untreated, VEGF<sup>165</sup> (100ng/ml; PeproTech, Rocky Hill, NJ), 200ng/ml PK2 (200ng/ml; PeproTech), PKRA7 (1µg/ml), VEGF<sup>165</sup> + PKRA7, or PK2 + PKRA7 in triplicate per condition. VEGF and PK2 were dissolved in water plus 0.1% BSA (water + 0.1%BSA used as control). Control, PK2, PK2 + PKRA7 or PK2 + 1% B6246 (anti-PK2 serum) were conditions used for IHMVEC cells in additional experiments. Three images were recorded of each well at each time point (8 hours). The number of connections between cells was counted, averaged and normalized. For MEECs,  $5 \times 10^4$  cells were plated and images were recorded at 6 hours, and for HMEC-1s,  $3 \times 10^4$  cells were plated and images were recorded at 8 hours.

## **2.4 Myeloid Cell Migration Assays**

### **2.4.1 Transwell Migration Assays**

The bottom chambers of 24-well transwell plates (8µm pore polycarbonate membrane transwell; Corning, Corning, NY) contained 600µl of each assaying

condition in complete media (RPMI +10% FBS, P/S for THP-1 cells) or minimal media (DMEM for RAW264.7 cells). For THP-1 cells, the bottom chambers of the transwells containing RPMI media were untreated or contained PKRA7 (1µg/ml), MCP-1 (100ng/ml; PeproTech), MCP-1 + PKRA7, PK2 (200ng/ml; PeproTech), or PK2 + PKRA7 in triplicate per condition. For RAW264.7 cells, the bottom chambers of the transwells containing DMEM media were untreated or contained PKRA7 (1µg/ml), SDF-1α (200ng/ml; PeproTech), SDF-1α + PKRA7, PK2 (200ng/ml), or PK2 + PKRA7 in triplicate per condition. MCP-1, SDF-1α and PK2 were all dissolved in water plus 0.1% BSA (water + 0.1% BSA used as control). The appropriate number of cells was collected in complete (THP-1) or minimal media (RAW264.7) and 100µl were plated onto the top chamber. The transwell plates containing the cells were placed in an incubator and the cells were allowed to migrate for 6 hours (THP-1) or 18 hours (RAW264.7). The cells were then fixed with 4% PFA, stained with 0.5% toluidine blue in 4% PFA, counted using a microscope and analyzed.

#### **2.4.2 Cytokine Array**

Cell culture: THP-1 cells were cultured as described [DeCoursey *et al.*, 1996; Kim *et al.*, 1996]. To induce differentiation, cells were incubated with 10ng/ml PMA (Sigma-Aldrich) for three days in 35-mm tissue culture dishes. The cells were washed with PBS to remove the PMA and then cultured in PMA-free media another 1 or 2 days. Then, recombinant human PK2 protein (200ng/ml) was added into the medium for 4 hrs. To examine the effect of PKRA7 on chemokine expression, the

THP-1 macrophages were pre-treated with 1 µg/ml PKRA7 for 0.5 h, then treated with 200ng/ml PK2 for 4 h.

qPCR array: Prior to amplification of cDNA, total RNA was isolated using TRIZOL reagent (Bethesda Research Labs, Gaithersburg, MD) according to the manufacturer's procedures. This is a modification of the acid-guanidinium-phenol extraction method of Chomczynski [Chomczynski, 1993]. The concentration of RNA in any sample was measured by spectrophotometry. Samples were stored at -70°C until they were used for reverse transcription. Reverse transcription (RT) was carried out using SuperScript® III Reverse Transcriptase (Invitrogen). qPCR-based array for detection of cytokines, chemokines and their receptors was achieved with gene-specific primers. All the mRNA levels ( $\Delta Ct$ ) were normalized to  $\beta$ -actin. Data of the mRNA level changes were shown as log<sub>2</sub> of the Ct value changes ( $\Delta\Delta Ct = \Delta Ct_{PK2-treated} - \Delta Ct_{Ctrl}$ ).

### **2.4.3 *In Vivo* Macrophage Migration Assay**

Subcutaneous tumors were grown in 8 nude mice as previously described, 4 mice receiving daily 20mg/kg PKRA7 IP injections, 4 receiving control injections. 30 days after cell inoculation,  $5 \times 10^5$  luciferase-labeled RAW264.7 cells were IP injected into each mouse. 24 hours later, mice were sedated with Ketamine plus Xylazine (100mg/kg plus 5-10mg/kg IP), IP injected with the luciferase substrate and imaged using the Xenogen Imager. Photos were taken and the luciferase signal at the site of the tumor was measured and analyzed.

## **2.5 Statistical Analysis**

Result values were expressed as means and SEM, and significance was established by one-way ANOVA. In all analyses, the level of statistical significance was 95% confidence level ( $p, 0.05$ ) and \* represents  $p \leq 0.05$ .

## **3. PKRA7 Inhibits Xenograft Tumor Growth of GBM and Pancreatic Cancer**

### **3.1 Introduction**

The tumor microenvironment is comprised of different cell types that function to contribute to cancer progression. These non-tumor cell types include myeloid cells of the immune system that can influence tumor progression in a variety of ways such as enhancing angiogenesis and tumor cell proliferation and motility [Kusmartsev and Gabrilovich, 2006]. The presence of myeloid cells in GBM and pancreatic cancer can contribute to aggressiveness and poor prognosis in these cancer types [Kurahara *et al.*, 2011; Roggendorf *et al.*, 1996]. Recent research demonstrates that myeloid cells produce and are regulated by a small peptide called PK2 and numerous studies have indicated that PK2 and its signaling pathway are important components of myeloid cell functions that contribute to tumor progression [Franchi *et al.*, 2008; Martucci *et al.*, 2006; Shojaei *et al.*, 2007b]. Studies have shown that treatment with anti-PK2 antibodies in mouse models of cancer can suppress disease progression, suggesting that the PK pathway is a viable target to investigate in our GBM and pancreatic cancer models [Shojaei *et al.*, 2008a].

The positive results from proof-of-principle experiments with neutralizing antibodies against PK2 have laid the foundation for further development of anti-PK2 agents into therapeutics. In this document, I report our findings on the anti-tumor activity of a synthetic small molecule PK2 antagonist, PKRA7 (prokineticin receptor antagonist 7) which can compete for the binding of PK2 to its receptors PKR1 and

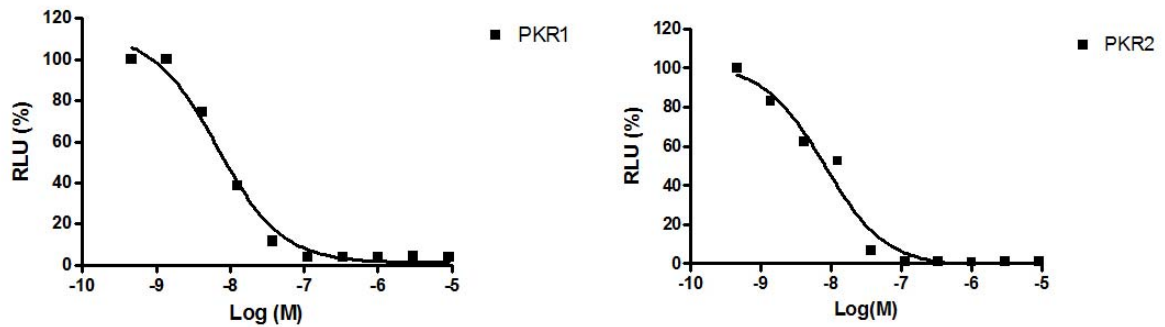
PKR2, consequently inhibiting the ability of PK2 to activate pro-tumorigenic downstream pathways in the tumor microenvironment.

### **3.1.1 Effect of PK2 Signaling Antagonist**

Because an anti-PK2 neutralizing antibody was found by previous studies to display anti-tumor activity, we explored the possibility that small molecules can be developed to achieve the same anti-tumor efficacy with lower costs and easier delivery [Imai and Takaoka, 2006]. The N-terminus of PK2 contains variations of the signal sequence for secretion, MRSLLILV, upstream of a sequence of amino acids that is completely conserved among all species, AVITGA [Li *et al.*, 2001; Tsuchiya *et al.*, 2003]. Mutation and deletion studies have shown that the AVITGA hexapeptide in the N-terminus region and the C-terminal cysteine-rich domain are important for functionality of prokineticins [Bullock *et al.*, 2004]. Mutant peptides with an A1M (alanine to methionine) substitution or addition of methionine to the N-terminus of PK1 resulted in a loss of biological activities from both PKRs. Calcium mobility activity of PKR1 and PKR2 stably expressed on Chinese hamster ovary (CHO) cells was significantly decreased when the aforementioned PK1 mutants were present. Since a previous study demonstrated that activation of PKRs can promote cell proliferation, a thymidine incorporation assay was performed in the presence of the N-terminal mutants on CHO cells stably expressing PKR1 and PKR2 [Lin *et al.*, 2002]. The presence of the PK1 mutants resulted in decreased [<sup>3</sup>H]thymidine incorporation indicating an inhibition of cell proliferation [Bullock *et al.*, 2004]. Following these observations, over 200 small molecule compounds have been

synthesized that structurally mimic the PK2 N-terminal region and PK1 mutants and tested for their ability to competitively inhibit the binding of recombinant PK2 to its receptors [Zhou, manuscript in preparation]. From this initial screen, over 60 water-soluble compounds were found to exhibit an inhibitory effect on PK2-receptor interaction with a binding constant below 20nM.

We have chosen compound PKRA7 for our experiments because it is able to bind to PKR1 and PKR2 with  $IC_{50}$  values of 5.0 and 8.2nM, respectively (Figure 3.1), and more importantly, it can penetrate the BBB due to its low molecular weight (around 400 daltons), a feature that could be critical for the treatment of GBM. The clearance rate of PKRA7 in mice was measured to be about 40 hours, allowing a schedule of daily delivery to the animals by IP injection.



**Figure 3.1 Potency of PKRA7 in antagonizing PKR1 and PKR2.**

Antagonist potency was examined in Chinese Hamster Ovary (CHO) cells that stably express PKR1 or PKR2. Inhibition of PKR1 or PKR2 activation by PK2/PK2 in the presence of different concentrations of PKRA7 was measured with a luminometer. RLU is an index for calcium influx measurement for this luminescence-based assay. The  $IC_{50}$  of PKRA7 for PKR1 and PKR2 were determined to be 5.0 and 8.2 nM, respectively.

### **3.1.2 Experimental Design for Testing PKRA7 Efficacy**

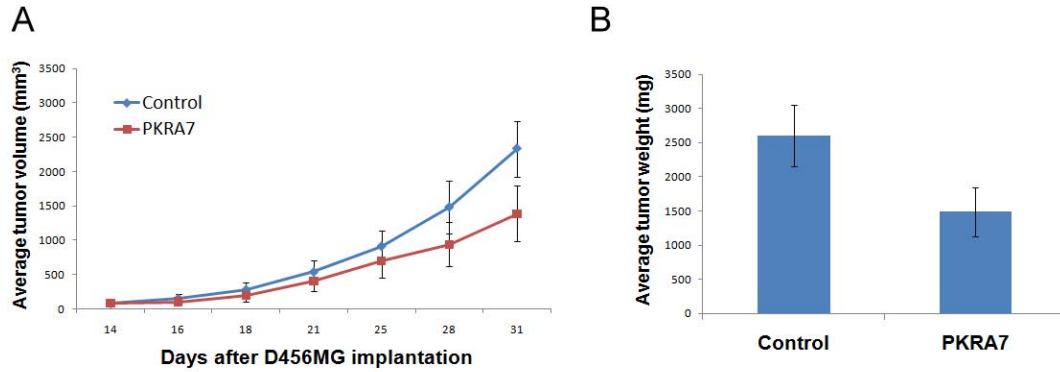
We decided to test PKRA7 in xenograft models of GBM and pancreatic cancer for our initial experiments. We hoped to determine whether PKRA7 could have an impact on tumor growth through its inhibitory effect on myeloid cell function. The xenograft tumor model offers a straightforward approach to assessing effects on tumor progression. In our models, nude mice were inoculated with human cancer cell lines SC on the flank and treated with PKRA7 or control by IP injection. Tumor growth can then be measured during the course of the experiment. Results from these experiments determined further investigation into the effects of PKRA7 on tumor progression.

## **3.2 Results**

### **3.2.1 PKRA7 Suppresses SC Tumor Growth in a Nude Mouse Xenograft Model of GBM**

To study the *in vivo* effect of PKRA7 on GBM tumor growth, we generated subcutaneous human GBM tumor xenografts in nude mice.  $5 \times 10^4$  D456MG glioma cells were implanted SC into ten nude mice and the mice were separated into two treatment groups beginning 14 days after implantation. The mice in the control group received an IP injection of PEG400 diluted 1:10 in PBS, while the mice in group 2 received IP injections of PKRA7 in the same PBS solution at a dose of 20mg/kg/day. Tumor sizes were monitored every three days and growth curves were generated (Figure 3.2A). 31 days after implantation the tumors were isolated after the mice

were sacrificed and weighed (Figure 3.2B). Mice treated with PKRA7 showed a trend of decreased tumor growth in both D456MG tumor growth rate and tumor weight.

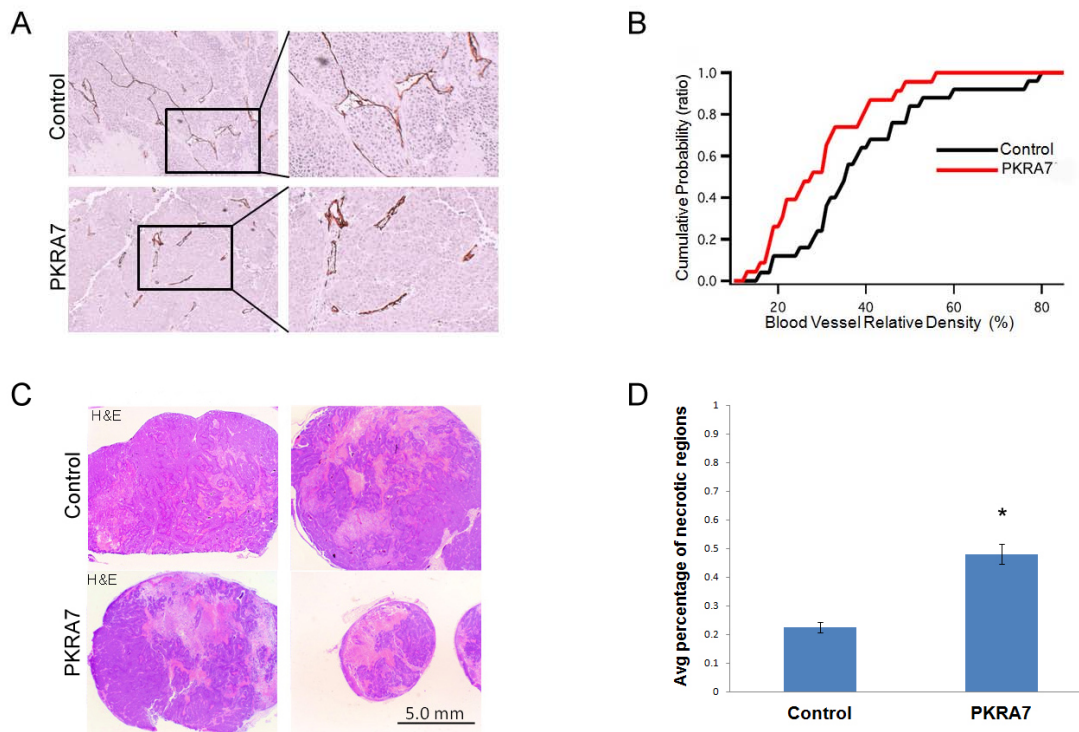


**Figure 3.2 PKRA7 decreases subcutaneous GBM xenograft tumor growth.**

(A) D456MG cells were SC injected into nude mice, and control (n=5) or PKRA7 (n=5) treatment was commenced when tumors became visually detectable (14 days). Measurements were taken every 2-3 days. (B) Average tumor weight of control and PKRA7-treated mouse tumors after removal.

### 3.2.2 PKRA7 Blocks Angiogenesis in GBM Xenograft Tumors

To determine the mechanism by which PKRA7 inhibited xenograft tumor growth, we quantified differences in blood vessel density and areas of necrosis in D456MG tumors treated or untreated with this compound. As shown in Figure 3.3B, we observed a decrease in relative blood vessel density as assessed by CD34 positive staining. Additionally, a significant increase in areas of necrosis of the PKRA7-treated tumors were observed in comparison to controls, suggesting that PKRA7 may suppress tumor formation primarily through inhibition of angiogenesis in a similar fashion to the PK2-neutralizing antibodies (Figure 3.3D) [Shojaei *et al.*, 2007b].



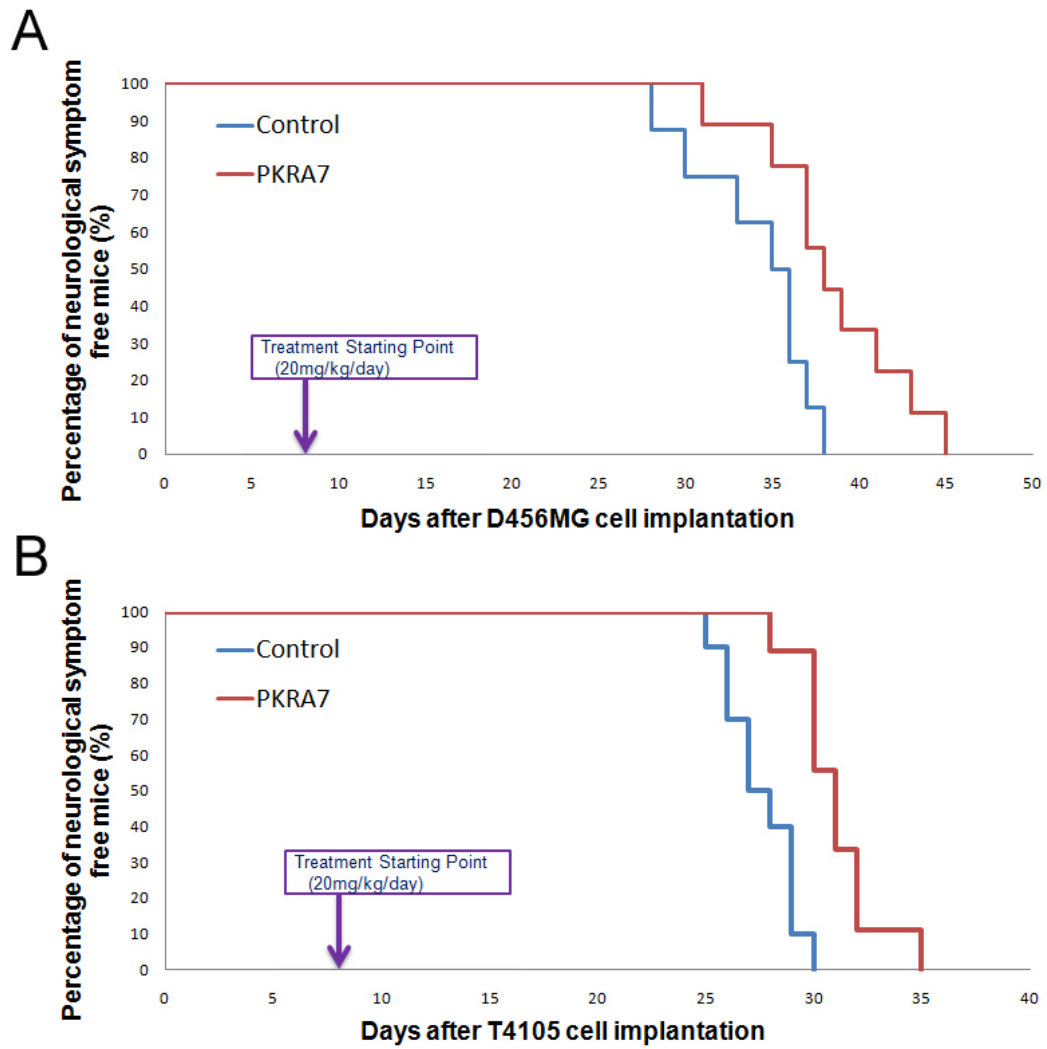
**Figure 3.3 PKRA7 leads to decreased vessel density and increased necrosis.**

(A) IHC staining using CD34 endothelial cell marker in D456MG SC tumors from mice treated with control or PKRA7. (B) Cumulative probability of vessel relative density as measured by CD34 staining. Vascular density of tumors decreased with PKRA7 treatment. (C) Representative pictures of H&E staining of sections from control and PKRA7-treated SC tumors (D) Quantification of necrotic regions from 5 slides of each tumor per treatment group, percentages of necrotic areas were measured by ImageJ (\* $p \leq 0.05$ ).

### 3.2.3 PKRA7 Prolongs Survival in Nude Mice after IC Xenograft Implantation of GBM Cells

Based on these promising results with the suppression of subcutaneous tumor formation by PKRA7, we employed intracranial inoculation of glioma cells to assess the ability of PKRA7 to inhibit tumor growth in a pathologically relevant setting. The initial experiment used 1,000 D456MG cells comparing PKRA7 and control was promising, but the D456MG cells were unsorted and thus took a very

long time to form tumors (control average ~65 days, PKRA7 average ~75 days), so we repeated the experiment using more cells to see results of the experiment in a timely fashion. This time,  $1 \times 10^4$  D456MG cells were inoculated into the mice and treatment started 7 days after with daily IP injections of PKRA7 or control. The mice were sacrificed when neurological signs of growing tumor burden became evident and we generated a Kaplan-Meier curve (Figure 3.4A). In this assay, treatment with PKRA7 significantly delayed the onset of neurological signs of tumor burden (mean survival of 38.4 days vs. 34.1 days for PKRA7 and control, respectively,  $p \leq 0.05$ ), indicating that PKRA7 was effective in inhibiting tumor growth in the IC environment. Similar results were obtained utilizing a second glioma cell line, T4105 with a mean survival of 31 days for mice treated with PKRA7 vs. 27.6 days for mice receiving control treatments ( $p \leq 0.05$ ), further demonstrating the efficacy of PKRA7 in the inhibition of GBM growth in a pathologically relevant setting (Figure 3.4B).

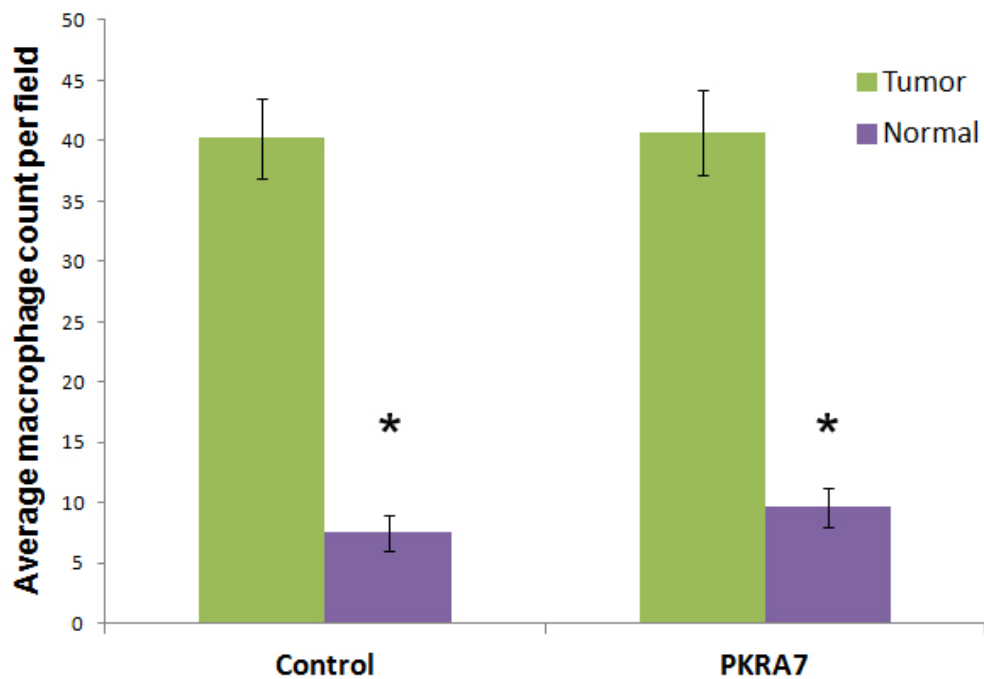


**Figure 3.4 PKRA7 decreases intracranial GBM xenograft tumor growth.**

(A)  $1 \times 10^4$  D456MG cells were IC injected into nude mice and treatment started 7 days after tumor implantation. Mice in control (n=8) or PKRA7 treatment (n=9) group were sacrificed when they developed severe neurological phenotype indicative of tumor growth intracranially. (B)  $1 \times 10^4$  T4105 cells were IC injected into nude mice and treatment started 7 days after tumor implantation. Mice in control (n=10) or PKRA7 treatment (n=9) group were sacrificed when they developed severe neurological phenotype indicative of tumor growth intracranially.

We examined the macrophage infiltration in the IC D456MG xenograft tumors in brains harvested from mice treated with PKRA7 or control. Importantly, these brains

were not harvested from the mice at the same time as we wait for signs of tumor burden to sacrifice the mice. The average survival for mice in the control group after IC injection was 34.1 days while mice treated with PKRA7 survived significantly longer, 38.4 days. As such, the sections analyzed represent the same endpoint, achieved at different times. With this in mind, it was not surprising to see there was no difference in the macrophage infiltration measured by murine F4/80 IHC staining in the control and PKRA7-treated sections. However, our analysis did reveal a distinct pattern in the F4/80 staining for both groups. The average number of F4/80-positive cells per field in the tumor areas was significantly higher compared to normal brain tissue (Figure 3.5).



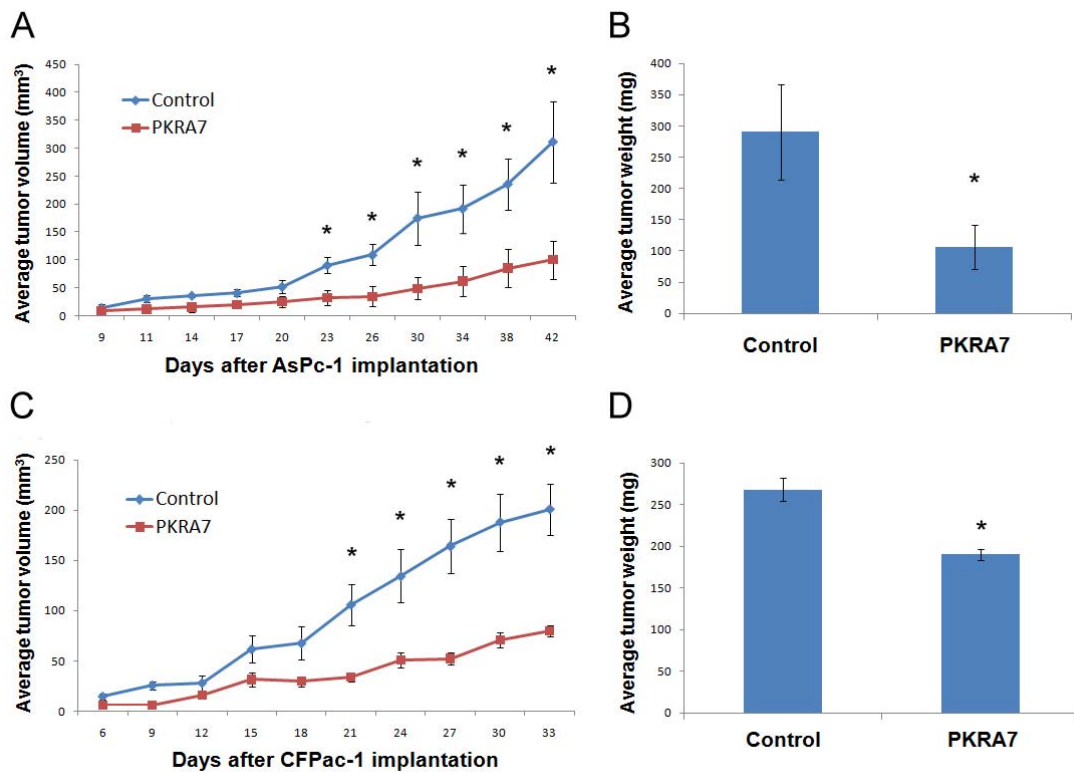
**Figure 3.5 Macrophage infiltration into GBM IC xenograft tumors**

Quantification of average macrophage infiltration of D456MG tumors in mice brains treated with control (n=2) or PKRA7 (n=2), 3 slides of each tumor per treatment group (\*p≤0.05).

These findings support reports that indicate myeloid cells present in the microenvironment can provide a source of PK2 to drive angiogenesis and contribute to tumorigenesis. While PKRA7 cannot completely block the presence of myeloid cells or angiogenesis in our GBM model, this treatment can inhibit these processes, reducing tumor growth rate and prolonging survival.

### **3.2.4 PKRA7 Suppresses SC Tumor Growth in a Nude Mouse Xenograft Model of Pancreatic Cancer**

Due to the well-established role of myeloid cells in the formation of pancreatic cancer, we next tested whether PKRA7 could have an impact on the xenograft growth of human pancreatic cancer cells in mice.  $5 \times 10^5$  AsPc-1 cells derived from the ascites of a patient with pancreatic cancer were inoculated SC into nude mice and daily PKRA7 or control treatment started 7 days after implantation. Growing tumors were measured every 2-4 days. As shown in Figure 3.5A, we observed suppression by PKRA7 on the growth rate of the AsPc-1 cells, resulting in a significant reduction in the average volume of the tumors. At the end of the experiment, the tumors were collected and weighed after the mice were sacrificed and we observed a significant decrease in tumor weight following treatment with PKRA7 (Figure 3.5B). Similar results were obtained with a different human pancreatic cancer cell line, CFPac-1 (Figure 3.5C,D).



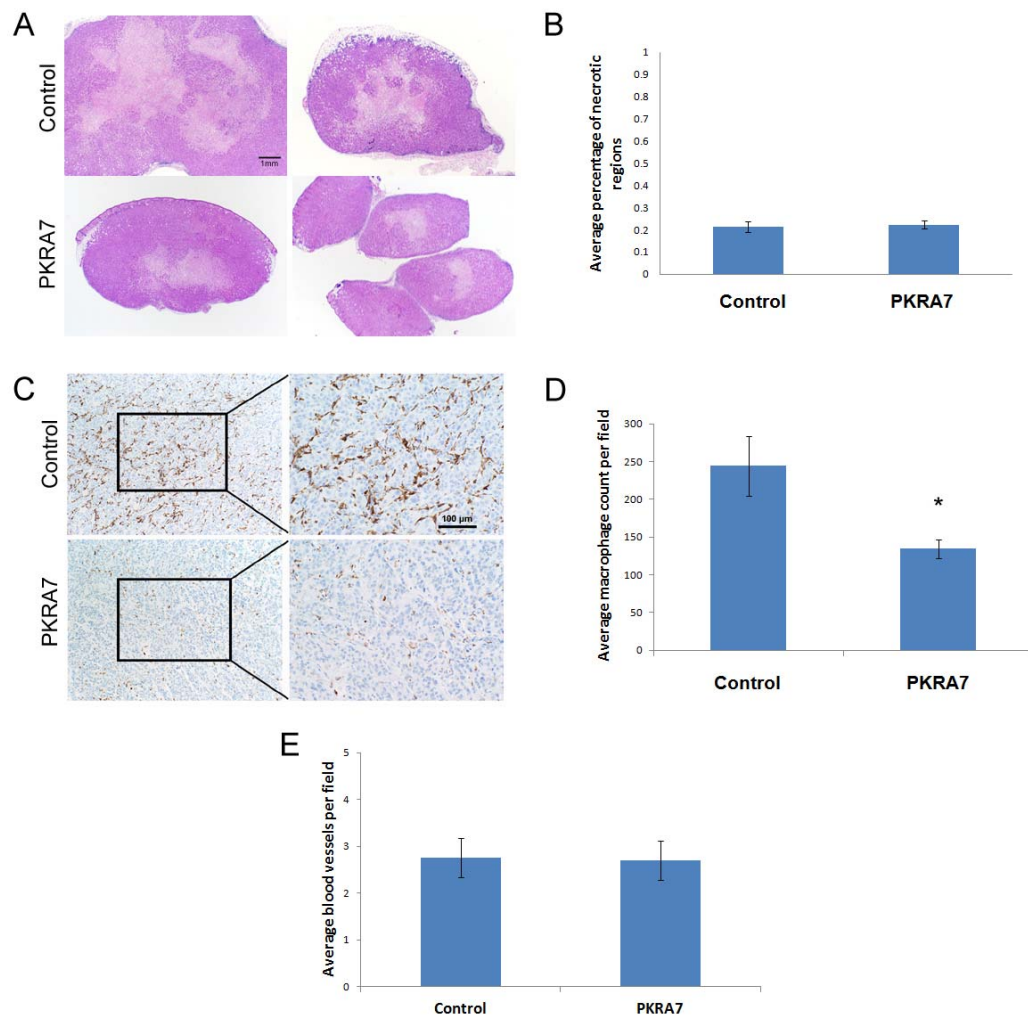
**Figure 3.6 PKRA7 decreases subcutaneous pancreatic cancer xenograft tumor growth.**

(A) AsPc-1 cells were SC injected into nude mice, and control (n=4) or PKRA7 (n=5) treatment was commenced when tumors were visible (9 days). Measurements were taken every 2-3 days. (B) Average tumor weight of control and PKRA7-treated mouse tumors after removal (\* $p \leq 0.05$ ). (C) CFPac-1 cells were SC injected into nude mice, and control or PKRA7 treatment was commenced when tumors were visible (6 days). Measurements were taken every 2-3 days (D) Average tumor weight of control and PKRA7-treated mice after tumor removal (\* $p \leq 0.05$ ).

### 3.2.5 PKRA7 Inhibits Macrophage Infiltration into Xenograft Pancreatic Cancer Tumors

To determine the potential mechanism underlying the significant reduction in tumor growth due to PKRA7 treatment, we examined tumor sections for signs of changes in angiogenesis and necrosis. There was no difference in the density of

blood vessels (Figure 3.6E) and similar levels of necrosis were observed for the tumors derived from PKRA7 and control treated mice (Figure 3.6B). In contrast, there was a significant decrease in the number of macrophages present in the tumors isolated from PKRA7-treated mice as measured by staining intensity of the mouse macrophage marker F4/80 (Figure 3.6D).



**Figure 3.7 PKRA7 inhibits macrophage infiltration into pancreatic cancer xenograft tumors**

(A) Representative H&E slides from control and PKRA7 treated tumors. (B) Quantification of necrotic regions from 5 slides of each tumor per treatment group, percentages of necrotic areas

were measured by ImageJ ( $p=0.205719$ ). (C) IHC staining using F4/80 mouse macrophage marker of AsPc-1 SC tumors treated with control or PKRA7. (D) Quantification of average macrophage infiltration of AsPc-1 tumors treated with control ( $n=4$ ) or PKRA7 ( $n=5$ ), 5 slides of each tumor per treatment group ( $*p\leq 0.05$ ). (E) Average number of CD34-positive blood vessels per field of view in AsPc-1 tumors, control ( $n=4$ ) or PKRA7-treated ( $n=5$ ), 5 fields per slide ( $p=0.928431$ ).

These results suggest that PKRA7 could inhibit pancreatic tumor growth via a different mechanism from that of GBM by blocking macrophage infiltration into the tumor microenvironment rather than suppressing angiogenesis. This observation is consistent with the phenotypic features of human pancreatic cancer as poorly vascularized but highly fibrotic and containing a large number of infiltrated myeloid cells, including macrophages [Korc, 2007].

### **3.3 Discussion**

Because of myeloid cell involvement in promoting GBM and pancreatic cancer tumor growth, we tested a small molecule antagonist, PKRA7, which disrupts the PK2 signaling pathway that is important for myeloid cell function. PKRA7 represents an exciting new therapeutic alternative for GBM and pancreatic cancer, two cancer types that currently have few effective treatment options. Our initial experiments using xenograft tumor models of these cancer types treated with PKRA7 showed decreased tumor growth compared to control treatment. Because PKRA7 is not a cytotoxic drug, it targets cells of the tumor microenvironment, such as myeloid cells and endothelial cells, inhibiting their ability to support tumor progression. Further analysis of the tumors derived from these *in vivo* experiments illustrated specific processes that were disrupted by treatment with PKRA7.

Analysis of xenograft GBM tumors showed histological features that indicated PKRA7 may be inhibiting tumor growth by disrupting of angiogenesis. CD34 IHC staining showed decreased blood vessel density and disorganized vessel structure in GBM tumors from mice treated with PKRA7 compared to control. There was also an increase in areas of necrosis in tumors from mice treated with PKRA7 compared to control. These increased areas of necrosis suggest a decrease in angiogenesis, leading to decreased oxygen and nutrient supply to tumor cells, resulting in tumor cell death. When we examined D456MG cells in an orthotopic xenograft setting using IC injections, survival was prolonged in mice treated with PKRA7 compared to control. Our data indicates that PKRA7 decreased tumor progression by blocking angiogenesis and other processes and increased the time to which mice exhibited signs of tumor burden.

Our *in vivo* experiments established the ability of a PKR antagonist to inhibit SC growth of xenograft pancreatic cancer cells in nude mice, demonstrating an important role for PK2 in pancreatic cancer tumorigenesis. We concluded that the role of PK2 was most prominent in macrophage recruitment and infiltration into the tumors. Pancreatic cancers are typically poorly vascularized and, consistent with this feature, we found no difference in vascular density or the area of necrosis in tumors from mice treated with control or PKRA7, indicating there was little difference in angiogenesis and oxygen supply to these two experimental groups. We did, however, observe a difference in staining for the mouse macrophage marker, F4/80, in the two treatment groups, suggesting the administration of PKRA7 blocked the migration and infiltration of macrophages into the growing tumors. This decreased

staining suggests that PKRA7 is able to block multiple processes of macrophage development and recruitment. PK2 is an important factor in myeloid progenitor cell differentiation and mobilization from the bone marrow [Shojaei *et al.*, 2007b]. Detecting fewer macrophages in the tumors suggests PKRA7 treatment may have reduced the number of macrophage cells present as well as their ability to mobilize and migrate towards the tumors.

The disruption of angiogenesis and macrophage infiltration in our *in vivo* tumor models of GBM and pancreatic cancer suggest PKRA7 may be a potent inhibitor against the signaling cascades that promote tumorigenesis. We wanted to further investigate the mechanisms by which PKRA7 was acting to drive the observed results. We next designed a series of *in vitro* assays to specifically test the different aspects of microenvironment contributions to tumor growth and to assess PKRA7's ability to inhibit these processes.

## 4. The Mechanism of Action of PKRA7

### 4.1 Introduction

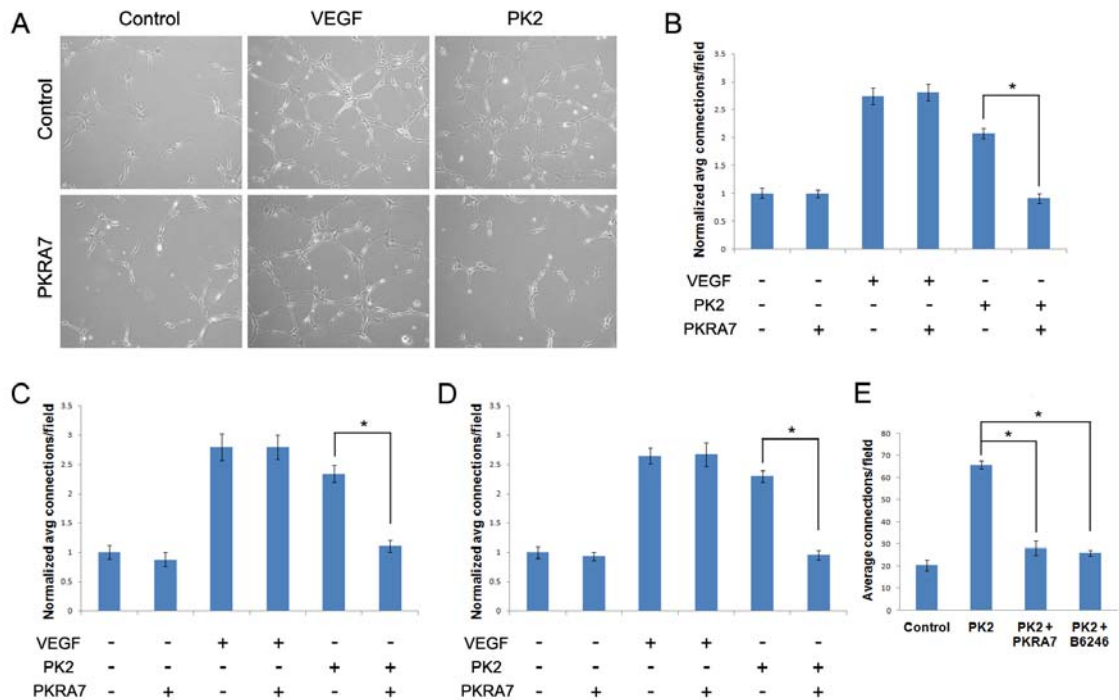
The results from the *in vivo* tumor xenograft studies of GBM and pancreatic cancer cells support the notion that the anti-tumor activity of PKRA7 is mediated via two different mechanisms. To further probe this observation at the cellular level, we conducted *in vitro* assays to assess the impact of PKRA7 on the angiogenic activity of endothelial cells, as well as the migratory ability of myeloid cells. A critical feature of angiogenesis is the sprouting of new blood vessels from existing ones [Hanahan and Weinberg, 2011]. This process can be experimentally observed using a capillary branching assay to measure connections between sprouting endothelial cells in the presence of known stimulators of angiogenesis such as VEGF and PK2 [Kerbel, 2008; LeCouter *et al.*, 2003]. The migration of myeloid cells like macrophages and monocytes is essential for their mobilization from the bone marrow to the tumor site and is mediated by known chemoattractants such as MCP-1, SDF-1 $\alpha$  and PK2 [Charo *et al.*, 1994; Shojaei *et al.*, 2007b; Wong *et al.*, 2001]. This migratory ability can be recapitulated in transwell migration assays to assess effects of different conditions and inhibitors, like PKRA7. Further, we shifted back to our *in vivo* system to measure the migration of exogenously introduced macrophages in mice towards a tumor site and how the presence of PKRA7 affects their migration. Finally, we tested the effects of PKRA7 on the expression of chemokine and chemokine receptor

mRNA by myeloid cells that may mediate migration or other pro-tumorigenic processes.

## **4.2 Results**

### **4.2.1 PKRA7 Inhibits Endothelial Cell Capillary Branching**

The ability of endothelial cells to form capillary tube-like network is an important indicator of angiogenesis [Donovan *et al.*, 2001]. To test whether PKRA7 could alter capillary formation, we employed immortalized human microvascular endothelial cells (IHMVECs). The cells were treated with 200ng/ml recombinant PK2 alone or PK2 plus 1µg/ml PKRA7 and plated onto a thin layer of Matrigel. As expected PK2 treatment induced capillary branching. However, as shown in Figure 4.1A, PKRA7 effectively inhibited PK2-induced capillary branching as measured by the number of connections between cells, but had no effect on VEGF-induced capillary branching (quantification presented in Figure 4.1B). Nearly identical results were obtained using mouse embryonic endothelial cells (MEECs) and primary human microvascular endothelial cells (HMEC-1) (Figure 4.1C, D). The specificity of the anti-PK2 activity in this assay was further confirmed by the demonstration of a similar effect by the anti-PK2 polyclonal antiserum (Figure 4.1E). Taken together, these results suggest that PKRA7 can specifically inhibit the angiogenic effect of PK2 on endothelial cells.



**Figure 4.1 PKRA7 blocks endothelial cell branching**

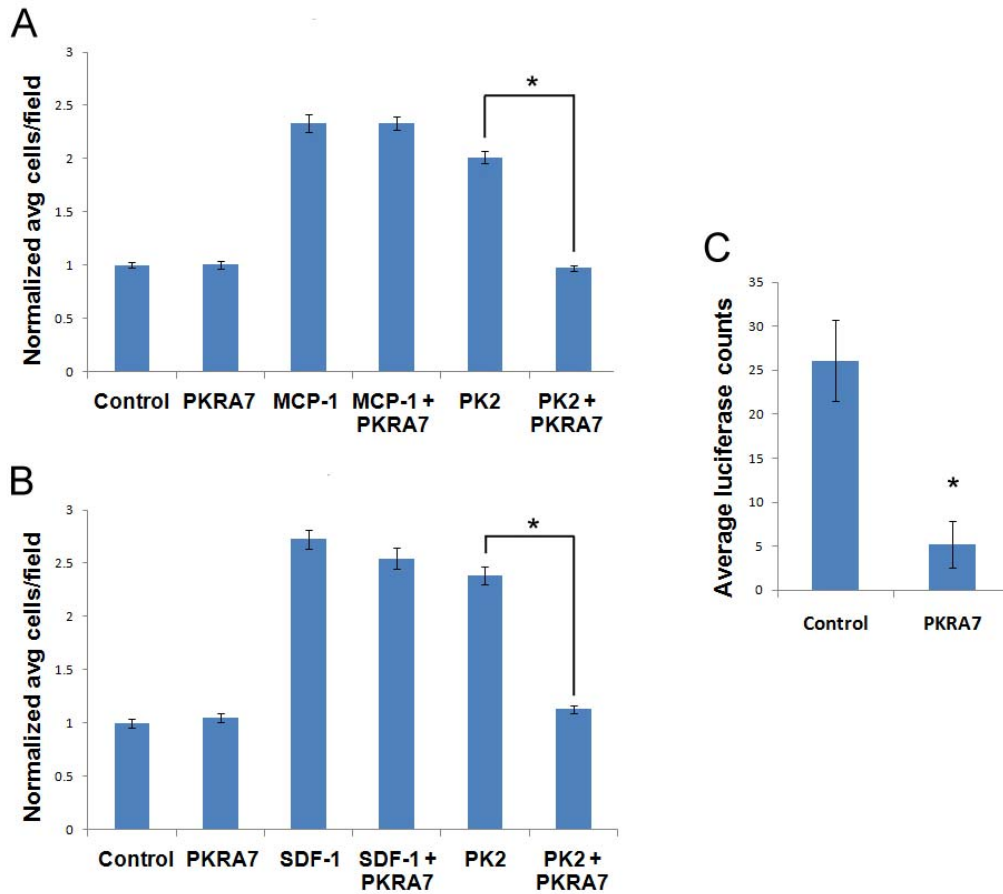
(A) IHMVECs were plated on Matrigel in the indicated treatment groups. Representative photographs were taken at 8 hours after plating. (B) Average number of connections between cells was counted and analyzed. Results are normalized data from 3 independent experiments. (C) Average number of connections between HMEC-1 cells was counted and analyzed. Results are normalized data from 3 independent experiments. (D) Average number of connections between MEEC cells was counted and analyzed. Results are normalized data from 3 independent experiments. (E) IHMVECs plated on Matrigel were untreated or treated with 200ng/ml PK2, 1% B6246 anti-PK2 serum, or PK2 + B6246 anti-PK2 serum. Representative photographs were taken at 4 hours after plating and analyzed (\* $p \leq 0.05$ ).

While these results support the importance of angiogenesis in GBM to support tumor growth and PKRA7's ability to block this process, PKR1 and PKR2 are not the only receptors on endothelial cells that contribute to angiogenesis. Given results from our *in vivo* experiments, the PKRA7 clearly plays a part in blocking angiogenesis but this may also be mediated by PKRA7's effects on overall myeloid

cell signaling, as they are important contributors to angiogenic signaling cascades including a variety of other pathways, examined in the experiments below.

#### **4.2.2 PKRA7 Inhibits Myeloid Cell Migration**

To determine the effect of PKRA7 on PK2-induced migration of myeloid cells, we employed the human monocyte cell line, THP-1, using a transwell migration assay. As predicted, PK2 induced migration of THP-1 cells towards the bottom chamber of the transwell. As shown in Figure 4.2A, 1 $\mu$ g/ml PKRA7 effectively impaired this PK2-induced migration of the THP-1 cells, but not the migration of those cells towards MCP-1, a known chemoattractant of THP-1 [Charo *et al.*, 1994; Wong *et al.*, 2001]. Similar results were observed with the mouse macrophage cell line, RAW264.7 with PKRA7 specifically blocking PK2-induced but not SDF-1 $\alpha$ -induced migration (Figure 4.2B). Therefore, PKRA7 specifically inhibits PK2-induced migration of myeloid cells from both human and murine origins. These results are consistent with PKRA7's blockade of myeloid cells into the tumor microenvironment observed in our *in vivo* experiments with xenograft pancreatic cancer tumors.



**Figure 4.2 PKRA7 blocks myeloid cell migration.**

(A)  $1 \times 10^5$  THP-1 cells on the top chamber of transwells were allowed to migrate for 4 hours. Cells were fixed, stained and the number of cells per field of view were counted. Results are the normalized average of 3 independent experiments. Addition of PKRA7 significantly blocked PK2-induced monocyte migration ( $*p \leq 0.05$ ). (B)  $7.5 \times 10^4$  RAW264.7 cells on the top chamber of transwells were allowed to migrate for 18 hours. Cells were fixed, stained and the number of cells per field of view counted. Results are the normalized average of 3 independent experiments. Addition of PKRA7 significantly blocked PK2-induced macrophage migration ( $*p \leq 0.05$ ). (C) Average measured luminescence of tumor site after IP injection of luciferase-labeled RAW cells into control (n=4) or PKRA7 (n=4) treated mice with SC AsPc-1 tumors 30 days after implantation. Average total luciferase counts were lower in mice treated with PKRA7 compared to control ( $*p \leq 0.05$ ).

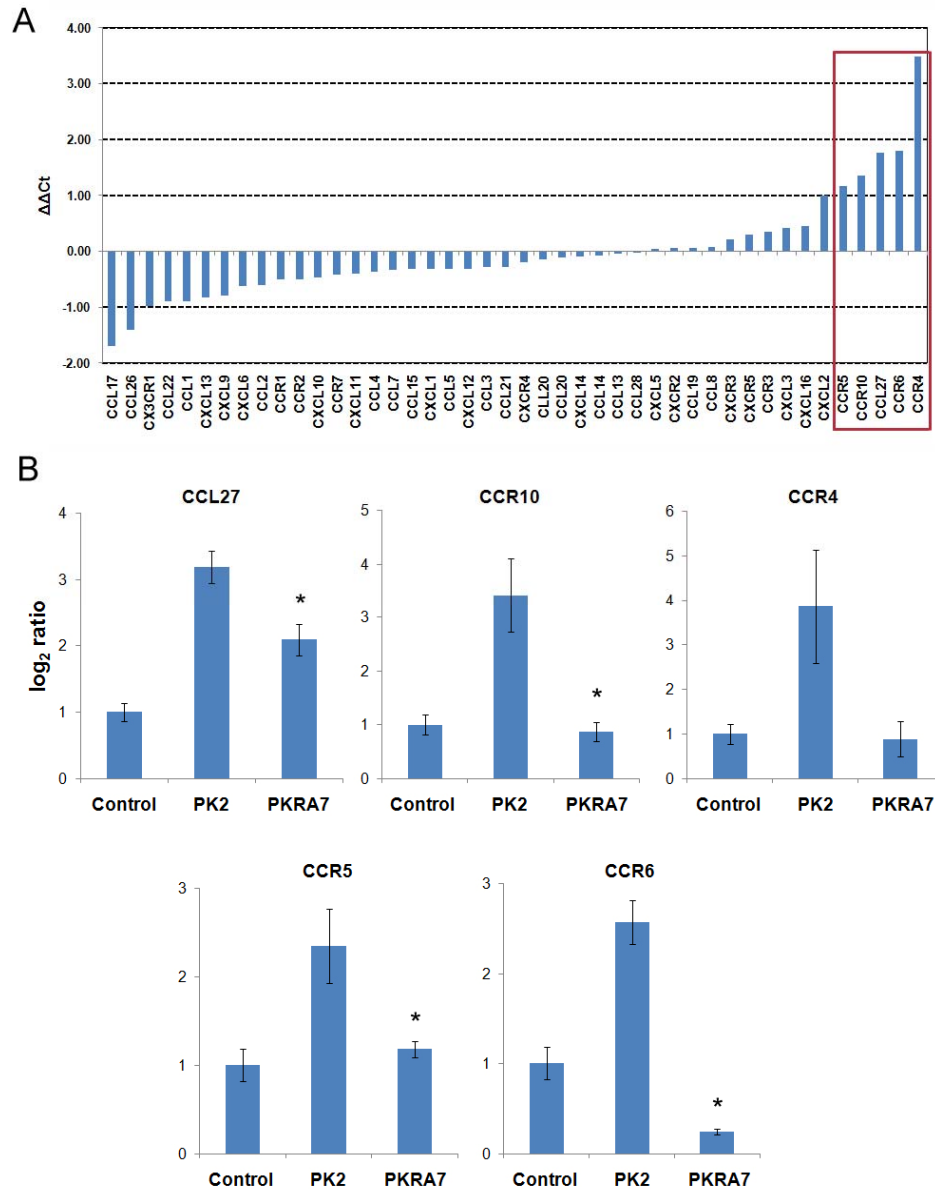
### **4.2.3 PKRA7 Blocks Macrophage Migration/Infiltration into Tumor Microenvironment in an *In Vivo* Setting**

To assess the impact of PKRA7 on migration/infiltration of mouse macrophages into the microenvironment of xenograft tumors formed by human pancreatic cancer cells, we IP injected luciferase-labeled RAW264.7 macrophage cells into nude mice 30 days after SC implantation of AsPc-1 cancer cells. We measured accumulation of RAW264.7 cells in the tumors 24 hours following their IP injection by recording the luciferase counts at the tumor site. As shown in Figure 4.2C, a significant decrease in luminescent signal emitted by the mouse macrophage cells was observed in mice treated with PKRA7 in comparison to that of the control mice. These results indicate that PKRA7 is able to block macrophage migration/infiltration into the tumor site in an *in vivo* setting, thus inhibiting the ability of the macrophages to positively contribute to the growth of xenograft tumors.

### **4.2.4 PKRA7 Blocks Upregulation of Pro-Migratory Chemokines Induced by PK2**

To further examine the mechanism by which PKRA7 blocks PK2-induced macrophage migration, we performed a quantitative-real time PCR cytokine array on RNA from THP-1 cells that were induced to differentiate into macrophage cells by PMA treatment. Among an array of 95 human chemokine ligands and their receptors, five displayed a significant induction in their expression after treatment with PK2 including CCL27, CCR10, CCR4, CCR5, and CCR6 (Figure 4.3A). At least four of these induced molecules are known to be involved in mobilization/migration of myeloid cells. Importantly, PKRA7 treatment attenuated the induction of each of

these molecules (Figure 4.3B), suggesting that suppression of the PK2-induced chemokines and receptors may represent the primary mechanism of anti-tumor activity of PKRA7 in the context of pancreatic cancer.



**Figure 4.3 PKRA7 blocks PK2-induced expression of specific chemokine/chemokine receptors.**

(A) qPCR-based array for detection of cytokines, chemokines and their receptors was achieved with gene-specific primers using THP-1 macrophages treated or untreated with PK2 for 4 h. All the mRNA levels ( $\Delta Ct$ ) were normalized to  $\beta$ -actin. Data of the mRNA level changes were shown as  $\Delta\Delta Ct = \Delta Ct_{PK2\text{-treated}} - \Delta Ct_{Ctrl}$ . Box region represents most highly upregulated genes that were used in further studies with PKRA7 as shown in lower panel. (B) qPCR assay to measure the effect of PKRA7 on the expression of chemokines and chemokine receptors that were identified to be induced by PK2 treatment. Data on the mRNA level changes were shown as  $\log_2$  of the Ct value changes. PKRA7 inhibits upregulation of CCL27, CCR10, CCR4, CCR5, and CCR6 (\* $p \leq 0.05$ ).

### 4.3 Discussion

We tested specific mechanisms that PK2 affects through a series of *in vitro* experiments that examine aspects of angiogenesis, macrophage migration and the cytokine production that controls these processes. We found that PKRA7 inhibited endothelial capillary branching, myeloid cell migration and pro-migratory chemokine expression by myeloid cells. These results offer a representation of how PKRA7 functions in the tumor microenvironment in our *in vivo* GBM and pancreatic cancer tumor models.

As discussed previously, angiogenesis is an important aspect of many cancer types, as it provides the oxygen and nutrient supply that is crucial for the survival and expansion of tumor cells. Many studies have shown that this process is especially vital in GBM [Furnari *et al.*, 2007; Louis, 2006]. While angiogenesis is a complex process that requires the interaction of many cell types, *in vitro* experiments using endothelial cells have been developed to easily manipulate stimuli and observe the consequences. We used a capillary branching assay to assess the impact of PKRA7 directly on endothelial cells. This experiment represents one central aspect of how PKRA7 can block tumorigenesis in GBM by disruption of angiogenesis. Our results indicated that PKRA7 successfully inhibited PK2-induced

capillary branching formation of three different endothelial cell lines. This inhibition was specific to PK2 as VEGF-induced capillary branching was not inhibited in these cells. While PKRs are important contributors to angiogenesis, there are other receptors that contribute to angiogenesis and blocking macrophage infiltration and/or macrophage signaling probably also plays a key role in inhibiting angiogenesis in GBM.

In our *in vivo* experiments with pancreatic cancer cells, we observed a decreased number of mouse macrophages present in tumors grown in mice treated with PKRA7 compared to control. This decreased infiltration suggested the inhibition of macrophage mobilization and migration. We chose to test PKRA7's ability to block macrophage migration in an *in vitro* setting using a transwell migration assay. We employed both a mouse macrophage (RAW264.7) and human monocyte (THP-1) cell line to assess the effects of PKRA7. Exposure to both SDF-1 $\alpha$  and PK2 recombinant proteins in the bottom chamber of the transwells resulted in migration of RAW264.7 cells through the transwell to the opposite side of the membrane. PKRA7 blocked PK2-induced migration but not SDF-1 $\alpha$ -induced migration. By using the THP-1 cells line, we were also able to assess the effect of PKRA7 on effect migration of myeloid progenitor cells, representing the mobilization and migration of CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid progenitor cells from the bone marrow and into circulation en route to the tumor microenvironment. Again, we saw similar results where PKRA7 blocked PK2-stimulated migration of the THP-1 cells but not MCP-1-stimulated migration. Migration of luciferase-labeled RAW264.7 cells to a xenograft pancreatic cancer tumor site in an *in vivo* setting was also disrupted by PKRA7 treatment

compared to control. We concluded that PKRA7 specifically targets the PK signaling pathway, including angiogenic potential of endothelial cells and migration of macrophages.

Next, we determined the potential impact of PKRA7 on the production of cytokines/chemokines by macrophages. Macrophages that are still able to migrate to and infiltrate a growing tumor may still be affected by the presence of PKRA7 and may not produce chemokines that contribute to different aspects of tumorigenesis. To address this, we treated THP-1 cells differentiated into macrophages with PK2 and PK2 plus PKRA7 and tested for the expression of a panel of chemokines and their receptors. Indeed, we found that PK2 induces the production of chemokines and receptors involved in the migration of myeloid cells and PKRA7 could block this induction. The chemokine receptors CCR10 and CCR4, known to respond to chemo-attractants CCL27, MCP-1, and CCL22, have been shown to be critical in inducing mobilization and homing of myeloid cells and leukocytes to the tumor site [Ishida and Ueda, 2006]. The chemokine receptor CCR6 and its ligand CCL20 (MIP-3 $\alpha$ ) have been implicated in dendritic cell migration and appear to be important for maintaining a normal level of the macrophage population since CCR6<sup>-/-</sup> mice showed decreased numbers of macrophage cells [Wen *et al.*, 2007].

Taken together with data showing the response of myeloid cells to PK2, it is clear that PKRA7 acts to suppress pancreatic cancer by blocking the ability of PK2 to induce myeloid cell mobilization from the bone marrow as well as PK2's direct effect on the tumor microenvironment through pro-migratory responses. These effects are not limited to pancreatic cancer as macrophage infiltration and the effects

of macrophage signaling on GBM tumor cells growth and migration are also important aspects of the complex tumor microenvironment.

The effects of PKRA7 on immune cells in the tumor microenvironment offer unique and exciting approaches to treating GBM and pancreatic cancer. PKRA7 is not, however, cytotoxic and would therefore be less effective as a single agent treatment in the context of drug therapy for GBM and pancreatic cancer. Recently, the most successful cancer treatment regimens include a combination of therapeutic agents to target a patient's specific needs to treat the complexities of their disease. To investigate the idea that PKRA7 may be a part of such a combination therapy, we decided to test PKRA7 in our *in vivo* models of GBM and pancreatic cancer in the presence of standard therapeutics.

## **5. Combination Therapy Xenograft Assays with GBM and Pancreatic Cancer**

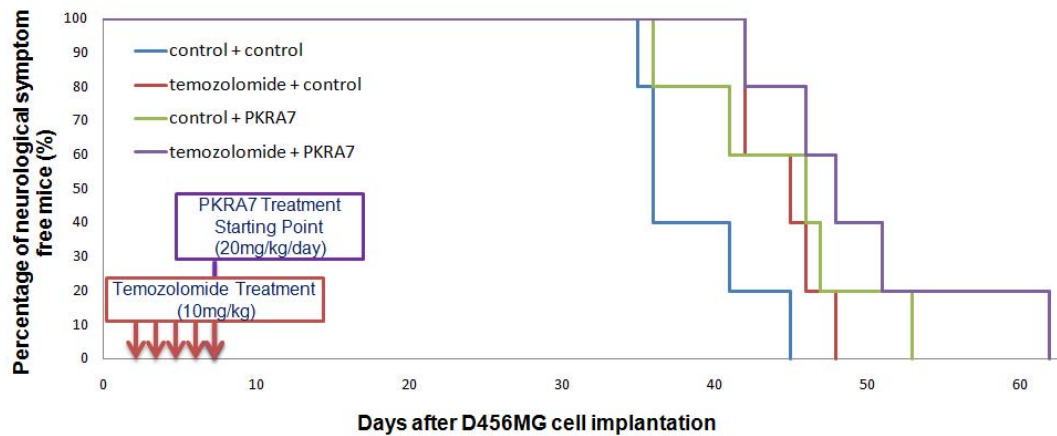
### ***5.1 Introduction***

Although PKRA7 displayed anti-tumor activities in the contexts of both GBM and pancreatic cancer, it is unlikely to be developed into a single-use therapeutic agent because it does not possess cytotoxic effects against tumor cells, a critical aspect of most cancer treatment regimens. Combination therapeutic strategies currently offer the greatest hope for effectively treating patients with aggressive cancers such as GBM and pancreatic cancer [Lane, 2006]. As a small molecule, PKRA7 represents a potential candidate for combination therapy treatments because of its advantages over current antibody-based treatments. As mentioned previously, small molecules offer benefits compared to therapeutic antibodies because small molecules can retain a high level of specificity for their targets while having lower manufacturing costs and a small size to allow crossing the BBB [Imai and Takaoka, 2006]. Previously, we have shown that treatment with PKRA7 can reduce xenograft tumor growth of GBM and pancreatic cancer cells. PKRA7 treatment also delayed the onset of neurological signs of tumor burden in mice with IC injections of xenograft GBM tumor cells. We decided to investigate a combination therapy approach in our mouse models of GBM and pancreatic cancer to test the ability of PKRA7 to synergize with standard chemotherapeutic agents.

## **5.2 Results**

### **5.2.1 PKRA7 Enhances the Efficacy of TMZ for GBM in a Xenograft Model**

The current standard of care for GBM is the chemotherapeutic agent, TMZ [Bei *et al.*, 2010; Stupp *et al.*, 2005]. To test whether PKRA7 could be effective in combination with TMZ, we examined the effect of this compound in combination with TMZ in our orthotopic model of GBM. Following an established experimental procedure for evaluating the effect of combinational therapy in xenograft mouse models [Cheng *et al.*, 2005; Patel *et al.*, 2000],  $1 \times 10^4$  D456MG cells were IC injected and the mice were treated with 10mg/kg TMZ or control for five days. For the remainder of the experiment, half of the mice received TMZ alone while the rest of the mice received TMZ plus PKRA7 and half of the control mice receive further control treatments while the remainder received PKRA7 treatments. As shown in Figure 5.1, treatment with both TMZ plus PKRA7 delayed the onset of neurological signs of tumor burden compared to mice receiving control, TMZ, or PKRA7 alone, indicating the trend of an enhanced effect of combinational therapy with the agents in inhibiting intracranial GBM growth in nude mice (mean survival of 49.8 days for PKRA7 plus TMZ vs. 44.6 days for either TMZ or PKRA7 alone vs. 38.6 days for control).



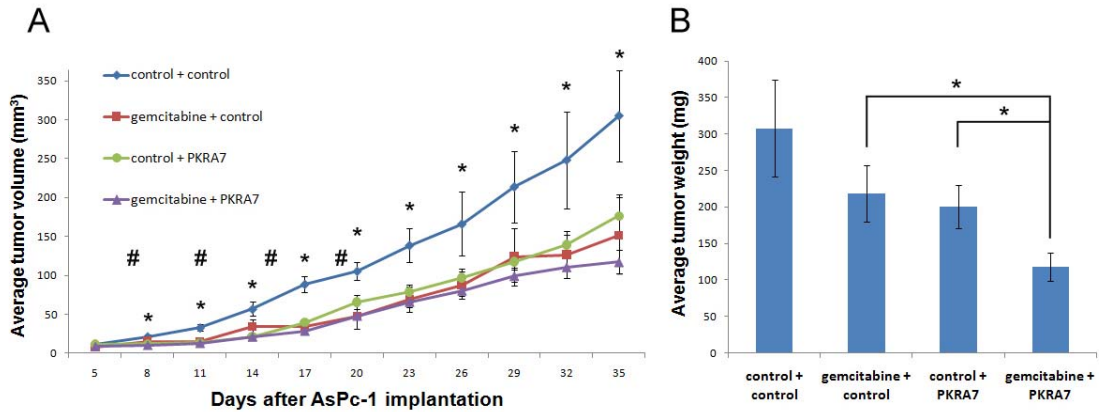
**Figure 5.1 PKRA7 enhances the efficacy of chemotherapeutic drugs to reduce GBM tumor growth.**

Kaplan-Meier curve of nude mice after TMZ and PKRA7 treatment following IC injection of  $1 \times 10^4$  D456MG cells. Treatment with 10mg/kg TMZ or control started 3 days after IC injection for a total of 5 consecutive daily treatments. Treatment with PKRA7 or control started 7 days after IC injection and continued for the duration of experiment. 5 mice per condition.

### 5.2.2 PKRA7 Enhances the Efficacy of GEM for Pancreatic Cancer in a Xenograft Model

For pancreatic cancer, GEM is one of the main chemotherapeutic drugs currently used in the clinic, and it was tested previously in combination therapy studies with the nucleoside analogue, troxacitabine, involving AsPc-1 cells [Damaraju *et al.*, 2007]. In our experiments,  $5 \times 10^5$  AsPc-1 cells were SC implanted into nude mice that were then treated with PKRA7 and GEM (100mg/kg) or control 7 days later. As shown in Figure 5.2B, tumors from mice that received both GEM and PKRA7 weighed significantly less than those from mice treated with only GEM or PKRA7. These results suggest that combinational therapy with GEM and PKRA7 is

more effective than the standard therapy or PKRA7 used alone in reducing pancreatic cancer xenograft tumor growth in our model.



**Figure 5.2 PKRA7 enhances the efficacy of chemotherapeutic drugs to reduce pancreatic xenograft tumor growth.**

(A) AsPc-1 cells were SC injected into nude mice, and control (n=10) or PKRA7 (n=10) treatment was commenced when tumors were visible (7 days). Treatment with 100mg/kg GEM (n=10) or control (n=10) started 7 days after tumor implantation and was administered every 4 days for two weeks for a total of 4 treatments (#). Measurements were taken every 3 days. 5 mice per condition. (\*p≤0.05 for control + control compared to GEM + PKRA7). (B) Average tumor weight of control, GEM, PKRA7 and GEM plus PKRA7-treated mouse tumors after their removal (\*p≤0.05).

### 5.3 Discussion

Combining cytotoxic chemotherapeutic agents with antibody or small molecules that target specific signaling pathways represent some of the most promising cancer treatment options. This type of therapy is currently being employed in many clinical trials and successful cancer regimens including combining the topoisomerase inhibitor irinotecan with the EGFR-targeting monoclonal antibody, cetuximab, to treat irinotecan-refractory metastatic colon cancer and combining doxorubicin, cyclophosphamide, paclitaxel with the HER2-targeting monoclonal

antibody, trastuzumab to treat HER2-positive breast cancer [Cunningham *et al.*, 2004; Romond *et al.*, 2005]. Like other therapeutics that are directed at signaling pathways targeting angiogenesis or immune cell functions, we theorized that PKRA7 would be a good candidate for use in combination therapy. After observing encouraging results in our *in vivo* models of GBM and pancreatic cancer using PKRA7 alone, we wanted to test whether these effects could be enhanced by combining the PKRA7 treatment with standard of care therapeutics for those cancer types.

Combining PKRA7 with the established chemotherapeutic treatments TMZ and GEM resulted in enhanced effects in our GBM and pancreatic cancer xenograft models, respectively. In our GBM model, the combination of PKRA7 and TMZ resulted in a trend of prolonged survival in mice with IC tumors compared to mice that received control treatment, PKRA7 alone or TMZ alone. In our pancreatic cancer model, combining PKRA7 and GEM resulted in smaller tumors compared to mice treated with control, PKRA7 alone or GEM alone. These results demonstrate the potential for developing this compound or similar derivatives as a component of combinational therapies. Researchers and clinicians have come to appreciate that a multi-pronged, tailored approach to cancer treatment is the most effective way to combat this deadly disease. Cancers that have been identified to be refractory or resistant to certain treatments like anti-VEGF therapies may require multiple therapeutic options to more effectively block the pathways involved in tumorigenesis. For example, patients with GBM may benefit from treatments blocking both VEGF and PK2 signaling pathways in combination with a chemotherapeutic therapy to

more effectively kill the tumor cells and decrease the contribution of the tumor microenvironment.

## 6. Conclusions and Future Directions

### **6.1 PKRA7 Inhibits Pro-Tumorigenic Processes in the Tumor Microenvironments of GBM and Pancreatic Cancer**

Tumorigenesis is a complex process that involves much more than proliferating tumor cells. The tumor cells are aided and supported by the surrounding stromal microenvironment that is rich with a heterogeneous mix of cells such as fibroblasts, endothelial cells and immune cells [Nyberg *et al.*, 2008]. These cells respond to signals from the tumor by secreting factors of their own that can perpetuate growth signals, remodel the extracellular matrix, contribute to angiogenesis and redirect the role of immune cells. Angiogenesis has long been understood to be an important part of tumorigenesis, and there is interest in blocking this process to inhibit tumor growth and metastasis [Ferrara, 2010]. While important angiogenic factors have been identified such as VEGF, therapeutics against the VEGF signaling pathway have not been proven to be universally successful. Indeed, many cancers are resistant to anti-VEGF therapy or become refractory to administration of these anti-VEGF treatments such as bevacizumab, resulting in recurrence that is sometimes more aggressive than the primary tumor [Casanovas *et al.*, 2005; de Groot *et al.*, 2010; Ebos *et al.*, 2009; Norden *et al.*, 2008; Paez-Ribes *et al.*, 2009; Rubenstein *et al.*, 2000; Shojaei and Ferrara, 2008b; Shojaei *et al.*, 2009].

There is a need to identify and target additional signaling pathways that may contribute to angiogenesis and other processes that have been shown to be important for tumor progression such as macrophage infiltration. PK2 and its

receptors are part of a signaling pathway involved in myeloid cell mobilization [Shojaei *et al.*, 2007b]. Multiple studies have shown that the CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid precursor cells can contribute to angiogenesis and tumorigenesis in a variety of cancer types [Shojaei *et al.*, 2008a; Shojaei *et al.*, 2009; Shojaei *et al.*, 2007b]. Macrophages derived from those precursor cells in the tumor microenvironment can also secrete cytokines that directly affect tumor cell growth. In recent studies, the anti-tumor efficacy of an anti-PK2 antibody has been found to be nearly as effective as an anti-VEGF antibody in preventing disease progression of a transgenic mouse model of pancreatic  $\beta$ -cell tumorigenesis, while the combination of the two antibodies showed an even more pronounced effect in inhibiting subcutaneous growth of several human cancer cell lines (colon cancer, rhabdomyosarcoma) and mouse tumor cells (mastocytoma, lymphoma) [Shojaei *et al.*, 2008a; Shojaei *et al.*, 2007b]. Anti-PK2 antibody treatment also reduced the number of circulating and tumor-infiltrating CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells, including bone marrow-derived macrophages, which have been shown to mediate refractoriness to anti-VEGF therapies in several mouse xenograft tumor models [Shojaei *et al.*, 2009; Shojaei *et al.*, 2007b]. Those studies have indicated that PK2 is a practical target for cancer therapy.

Antibody-based therapies represent a significant portion of cancer treatment options in today's clinics. However, studies with patients and mouse models of GBM and pancreatic cancer have shown that these types of cancer can be resistant or refractory to anti-VEGF signaling antibody therapies [Casanovas *et al.*, 2005; de Groot *et al.*, 2010; Norden *et al.*, 2008; Paez-Ribes *et al.*, 2009; Rubenstein *et al.*, 2000; Shojaei *et al.*, 2009]. Other studies have shown that this resistant response

may be common to additional types of cancer such as breast and colon cancer [Dempke and Heinemann, 2009; Ebos *et al.*, 2009; Takahashi and Nishioka, 2011]. Patients may therefore benefit from additional therapies that target alternate pathways in combination with anti-VEGF signaling therapies to prevent refractory responses. Thus, small molecule inhibitors offer an alternative therapeutic approach because they can still be specific to their targets while being more cost effective to manufacture [Imai and Takaoka, 2006]. Also, some drug therapies are required to cross BBB to treat diseases such as GBM, and small molecule inhibitors are good candidates for this purpose due to their small size. In this regard, our demonstration that PKRA7 is capable of penetrating the BBB in the mouse and acts to inhibit IC xenograft tumor formation by GBM cells demonstrates an alternative strategy to inhibit tumor angiogenesis that contributes to tumor progression.

Desmoplastic stroma is a defining feature of pancreatic cancer and can contain high levels of TAMs, especially at the tumor/stroma interface of pancreatic cancer where the tumor invades the surrounding normal tissue [Korc, 2007; Kurahara *et al.*, 2011]. This infiltration of macrophages is thought to contribute to disease progression by releasing growth factors and cytokines that promote tumor cell invasion and suppress antitumor immunity, and this infiltration is associated with poor prognosis [Kurahara *et al.*, 2011]. Recent studies report that immunosuppressive cells including TAMs, myeloid-derived suppressor cells (MDSCs) and regulatory T cells ( $T_{reg}$ ) were found in high levels in early to late stages of progressing cancer compared to normal tissue in a mouse model of pre-invasive and invasive PDA [Clark *et al.*, 2007]. PK2 may play a critical role in the complex

and dynamic relationship between pancreatic cancer cells and these stromal cells through the regulation of recruitment and activity of myeloid cells. This role for PK2 is supported by the results from our *in vivo* experiments demonstrating decreased pancreatic cancer xenograft tumor growth after treatment with PKRA7.

Results from our *in vivo* experiments with GBM and pancreatic cancer models were encouraging and led to further investigating the mechanism of action of PKRA7 using *in vitro* models. To measure angiogenesis in an *in vitro* setting, a capillary branching assay was performed with three different endothelial cell lines including human and murine lines. The assay demonstrated PKRA7's ability to specifically block PK2-induced capillary branching, and we believe this can partly explain why we observed features of disrupted angiogenesis in GBM tumors from mice treated with PKRA7. Next, we looked at the migration of myeloid cells, both progenitors and differentiated macrophages, using a transwell migration assay. Results from our experiments with xenograft pancreatic cancer tumors showed decreased macrophage infiltration into tumors from mice treated with PKRA7. PKRA7 also specifically blocked PK2-induced migration of THP-1 and RAW264.7 cells in our transwell migration assay, corroborating the results seen in the tumor sections. We also performed an *in vivo* migration assay where luciferase-labeled RAW264.7 cells were IP injected into AsPc-1 tumor-bearing mice receiving control or PKRA7 treatment. After 24 hours, the luciferase signal at the tumor site was measured and found to be significantly decreased in mice receiving PKRA7 treatment compared to control. Again, these findings demonstrate the ability of PKRA7 to block the migration and infiltration of macrophages to tumor cells. Finally, we examined a

panel of chemokines and receptors expressed by THP-1 cells in response to PK2 and assessed the ability of PKRA7 to block their expression. We found that PK2 induced expression of chemokines and receptors that promote migration and can control myeloid cell population levels and that PKRA7 can inhibit expression of these chemokines and receptors. Taken together, our *in vitro* assays validate many of the findings from our *in vivo* experiments and provide greater insight into PKRA7's mechanism of action in blocking tumor progression through effects in the tumor microenvironment. While these results are encouraging, we hypothesized that PKRA7's effects would be even more potent in combination with cytotoxic agents and decided to test this in our xenograft tumor models.

Combination therapies represent the future of cancer treatments by targeting multiple aspects of tumor progression. In our studies, we combined PKRA7 treatment with chemotherapeutic agents that are used as the standard of care therapeutics in GBM and pancreatic cancer. In both types of cancer, we found PKRA7 had a synergistic effect. Mice with IC injections of D456MG cells treated with both PKRA7 and TMZ had prolonged mean survival compared to mice receiving only PKRA7 or TMZ or control treatments. Likewise, AsPc-1 tumors from mice treated with both PKRA7 and GEM were smaller and weighed significantly less than tumor from mice receiving only PKRA7 or TMZ or control treatments. These results are encouraging for future experiments with PKRA7 within the laboratory and in the clinic. PKRA7's unique target makes it ideal for use in combination therapies that can be tailored more specifically for a patient's needs, increasing the chance for

prolonged survival and decreased resistance or refractory responses to anti-angiogenic therapies.

Ultimately, our goal for these studies is to test PKRA7, or compounds similar to it, in clinical trials with patients with GBM or pancreatic cancer to explore the advantages of targeting the prokineticin pathway in patients with cancer. An initial Phase I clinical trial would test the toxicity of PKRA7 in patients. Past pharmacokinetic experiments with PKRA7 in mice suggest that PKRA7 is well tolerated at levels required to saturate circulation and effectively antagonize PKRs. Further trials that could test the combination of PKRA7 with a standard of care therapeutic in patients would test PKRA7's ability to synergize and effectively block tumor progression in patients with few treatment options. Even prolonging survival by a few months can be considered an accomplishment for most patients with GBM or pancreatic cancer. As more is discovered about the contributions of the tumor microenvironment to these diseases, more effective treatments can be researched and implemented. We believe PKRA7 and the PK pathway will play an important role in these discoveries.

## ***6.2 Future Directions***

### ***6.2.1 The Effects of PKRA7 In Vivo***

Our experiments have demonstrated that antagonizing the PK pathway can negatively affect angiogenesis and macrophage infiltration, decreasing GBM and pancreatic cancer xenograft tumor growth. In the context of macrophage infiltration,

our results reveal that this process is important for both GBM and pancreatic cancer (Figures 3.5 and 3.7C, D). However, it is not clear how PKRA7 affects myelopoiesis in our models and the extent to which any effect on myelopoiesis may contribute to decreasing tumor growth. Indeed, PKRA7 could potentially block myelopoiesis in the bone marrow leading to fewer macrophages being available to mobilize from the bone marrow and infiltrate the tumors. Further experiments can be conducted to examine the effects of PKRA7 on myelopoiesis by examining myeloid cell populations in the bone marrow and circulation from mice treated with PKRA7 and control. Mice that receive whole-body radiation require myelopoiesis in the bone marrow to repopulate their immune system. Administering PKRA7 after radiation treatment and examining the resulting myeloid cell populations compared to mice receiving control treatments could establish PKRA7's effect on myelopoiesis.

Our experiments in the GBM model focused on angiogenesis and PKRA7's ability to inhibit this process. As mentioned previously, however, this process is almost certainly mediated by the presence of myeloid cells producing proangiogenic factors such as PK2. Further assays can be performed determine which process may be more important and which is affected most by PKRA7. Similar experiments to those previously mentioned can be conducted in mice that have myeloid lineages knocked out to distinguish whether the presence of myeloid cells is necessary for tumor growth and whether PKRA7 can still effectively block angiogenesis and GBM tumor growth in those mice lacking myeloid cells.

## 6.2.2 Alternative PKRA7 Delivery Methods

Through our continuing collaboration with Dr. Zhou, we have devised different methods of delivery for PKRA7 and its derivative compounds (discussed below). The daily IP injections of PKRA7 dissolved in PBS is simple yet time consuming and less practical for clinical trials. Initial experiments have investigated the use of oil solvents such as sunflower seed oil and ethyl oleate. PKRA7 can be dissolved in ethyl acetate and then in the oil at a concentration of 50mg/kg and injected SC into nude mice at the back of the neck. The mice continuously absorb PKRA7 from the oil under their skin. Pharmacokinetic studies have shown that the circulating levels of PKRA7 remain high even after 5 days, during which another dose is SC injected to maintain the circulating levels of PKRA7. This method is less time consuming while remaining precise in its dosage delivery and future experiments will investigate how this method compares to IP injections.

As some of the derivative compounds are more stable gastrointestinally than PKRA7, an oral gavage approach has also been explored. Oral delivery is one of the easiest methods to administer in a clinical trial, so we intend to employ this delivery method in our xenograft models in future experiments.

## 6.2.3 PKRA7 Derivative Compounds

As mentioned above, pharmacokinetic studies have started with compounds similar to PKRA7 that also have low  $IC_{50}$  values when antagonizing PKRs. These compounds, cpd41, cpd42 and cpd42R (a chiral form of cpd42), are more

metabolically stable than PKRA7 and can remain in circulation at high levels for a longer period of time. An oral gavage delivery method is being developed for cpd41 as it is the most gastrointestinal stable compound. However, initial experiments suggest daily administration is still required. An oral delivery method would be more tolerable in clinical studies so we plan to evaluate the efficacy of this delivery with cpd41 in our xenograft tumor models. Cpd42 and cpd42R are the most metabolically stable compounds and are ideal for studies using SC injection of these compounds in an oil solvent.

#### **6.2.4 Transgenic Mouse Model of Pancreatic Cancer**

Xenograft experiments with human cell lines and nude mice can be very useful during initial investigations. They are easily performed and usually offer timely results. However, transgenic mouse models represent a gold standard in cancer research using model systems for many reasons. Firstly, transgenic mouse models can include orthotopically relevant tumor formation due to genetic manipulation that can recapitulate the human disease. Also, the use of immune-competent mice can be useful and more accurately explore immune cell interactions in models of spontaneously arising tumors [Qian and Pollard, 2010]. Such a model for ductal adenocarcinoma of the pancreas (PDA) has been developed on a C57BL/6 background [Hingorani *et al.*, 2003; Hingorani *et al.*, 2005]. The three genes manipulated in the model are the tumor suppressor p53, the oncogene Kras and pancreas-specific gene PDX-1. Mutated p53 (Trp53<sup>R17H</sup>) and activated Kras (Kras<sup>G12D</sup>) are under the control of a Cre-Lox system while PDX-1 is fused with the

Cre protein (PDX-1-Cre). The resulting transgenic mice express Trp53<sup>R17H</sup> and Kras<sup>G12D</sup> only in the pancreas and over time develop PanINs (pancreatic intraepithelial neoplasias), precursor lesions leading to PDA. KC (LSL-Kras<sup>G12D/+</sup>;PDX-1-Cre) and KPC (LSL-Kras<sup>G12D/+</sup>;LSL-Trp53<sup>R17H/+</sup>;PDX-1-Cre) mice have been thoroughly studied and characterized. These mice represent an appealing model to study with PKRA7 and its derivative compounds. The KC mice develop PanIN lesions at 24-40 weeks (7-10 months) of age, and a few progress spontaneously to invasive and metastatic PDA. The KPC mice develop significant disease burden faster at around 10 weeks of age. The development of PDA is well documented by Hingorani *et al.* and offers guidelines for designing experiments to assess how PKRA7, in combination with GEM, could inhibit the progress of PanIN in KC or PDA in KPC mice. We are currently breeding these mice to obtain KC, KPC and wildtype littermates to use in upcoming experiments with PKRA7 and its derivative compounds. This transgenic model offers an unparalleled opportunity to study the contribution of the PK signaling pathway to pancreatic cancer progression.

### **6.3 Concluding Remarks**

In this work, we have explored the contribution of the tumor microenvironment to cancer progression in the context of GBM and pancreatic cancer. In our studies, we have focused on the presence of myeloid cells and their roles in tumorigenesis in these cancer types. We focused on the secreted regulatory peptide PK2 and the function of this signaling pathway in myeloid cell mobilization. Previous studies have characterized the role of CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells and PK2 pathway signaling in

various cancer models. Our goal was to assess the efficacy of a small molecule inhibitor of this pathway, PKRA7 in blocking tumor growth in models of GBM and pancreatic cancer. We showed that treatment with PKRA7 can effectively prolong the survival of nude mice with IC injections of GBM xenograft tumors and reduce the tumor size of SC grown GBM and pancreatic cancer xenograft tumors in nude mice. Also, IHC analysis of those SC tumors revealed evidence about the mechanism of action of PKRA7 in blocking tumor growth. In GBM tumor samples, we found evidence that angiogenesis was disrupted by treatment with PKRA7 as there were increased areas of necrosis and decreased blood vessel density observed in the tumor sections. In contrast, there were no differences blood vessel density or areas of necrosis in the pancreatic cancer tumor sections but a decrease in macrophage infiltration was observed in tumors from mice receiving PKRA7 treatment.

We decided to further investigate these mechanisms of action through a series of experiments aimed at reconstructing angiogenic and myeloid cell migration processes in *in vitro* settings. In a capillary branching assay used to mimic endothelial cell branching in angiogenesis, we found that treatment with PKRA7 blocked PK2-induced but not VEGF-induced capillary branching. We also found that PKRA7 inhibited monocyte and macrophage PK2-induced migration in a transwell migration assay. Examining a panel of chemokine and chemokine receptors revealed that PKRA7 could block expression of PK2-induced chemokine and receptor genes that promote migration, further explaining the decrease in macrophage infiltration seen in the pancreatic cancer tumors from mice treated with PKRA7. This observation was further confirmed using an *in vivo* migration assay

where luciferase-labeled macrophages were IP injected into mice bearing xenograft pancreatic cancer tumors receiving either control or PKRA7 treatment. After 24 hours, the measured luciferase count revealed a lower presence of macrophages at the tumor site in mice receiving PKRA7 treatment compared to control. The specificity of PKRA7 to target the PK pathway is promising for its potential use in the clinical setting. To reproduce the conditions of the clinic, we also tested PKRA7 in combination therapy experiments.

For our combination therapy experiments, we used both cytotoxic, standard of care therapeutics and PKRA7 in our GBM and pancreatic cancer models. When PKRA7 treatment was combined with TMZ in our GBM model, we observed a delayed onset of neurological signs of tumor burden compared to using either agent alone. In our pancreatic cancer model, we detected significantly smaller tumors in mice receiving both PKRA7 and GEM compared to mice treated with either one alone. From these observations, we concluded that PKRA7 can successfully synergize with other therapeutic options and could be a viable option to add to the treatment of GBM or pancreatic cancer. Indeed, our future goals for this project include further experiments with delivery methods for PKRA7 as well as derivative compounds that may be more metabolically stable than PKRA7 but target the same pathway. Eventually, we would hope to see a small molecule PKR antagonist in clinic trials for GBM or pancreatic cancer as there are presently so few treatment options for these cancer types. We believe the PK pathway represents an important contributor to the tumor microenvironment and have shown that it can be effectively targeted using our small molecule antagonist, PKRA7. The PK signaling pathway

and other contributors to tumor progression through modulation of the tumor microenvironment represent targets for researchers and clinicians pursuing novel cancer treatment options.

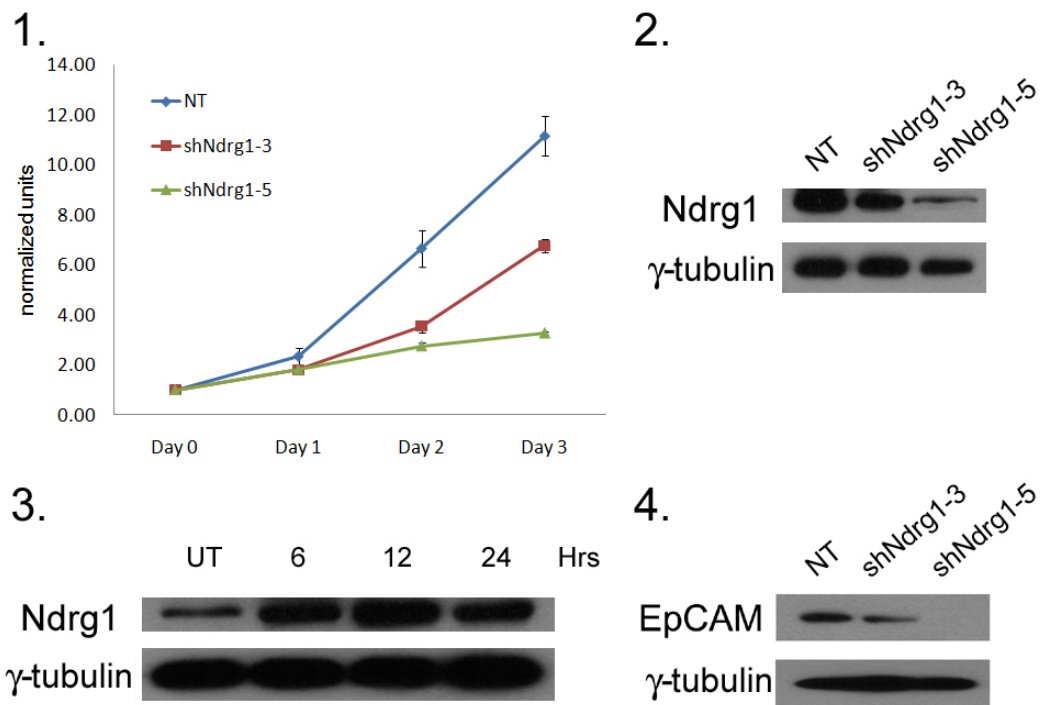
## Appendix

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer mortality [Kumagi *et al.*, 2009]. One of the most common complications with HCC is recurrence. There is great interest in identifying novel molecules that may functionally contribute to HCC tumorigenesis and recurrence. In our lab, we have become interested in the NdrG family of molecules, specifically NdrG1 (N-myc downstream regulated gene 1). NdrG1 is a 43kD protein that is ubiquitously expressed and has been shown to be mostly cytoplasmic in cells [Kovacevic and Richardson, 2006; Lachat *et al.*, 2002; Melotte *et al.*, 2010; Zhou *et al.*, 2001]. The NdrG proteins have no specific functional domains that have been identified though they appear to have a non-functional  $\alpha/\beta$  hydrolase domain that lacks essential amino acids that would confer enzymatic activity [Shaw *et al.*, 2002]. NdrG1 is unique from the other members of the family, however, because its sequence encodes three tandem repeats of a ten amino acid hydrophilic sequence at its C-terminus that no other members have. This sequence may confer important functionality for NdrG1 [Kovacevic and Richardson, 2006].

In previous studies NdrG1 has been identified as a possible tumor metastasis suppressor in a number of cancers such as breast, prostate, pancreatic, lung and colon cancer [Kovacevic and Richardson, 2006]. Previous literature and our preliminary data seem to suggest that NdrG1 may play a contrasting role in HCC, promoting tumorigenesis and recurrence. Using clinical samples, a recent study found that *NdrG1* mRNA was higher in HCC samples compared to normal and tissue adjacent to HCC. This study also found that increased NdrG1 expression correlated

with decreased patient survival [Chua *et al.*, 2007]. Another study showed increased IHC staining of NdrG1 in moderately to poorly differentiated HCC tissue samples compared to well-differentiated HCC. This study concluded that high NdrG1 expression may be associated with decreased survival [Akiba *et al.*, 2008].

Our experiments have shown that NdrG1 is highly expressed in more aggressive HCC cell lines compared to less aggressive and hepatoblastoma cell lines. Knockdown of NdrG1 in HCC cell lines using short hairpin RNA (shRNA) resulted in decreased cell viability as measure by a MTS assay (Figure A1). Additionally, we have found that NdrG1 expression is upregulated by ionizing radiation (IR) treatment (Figure A3). It is hypothesized that a cancer stem cell (CSC) population may contribute to the repopulation of a tumor after treatment and eventually recurrence [Gupta *et al.*, 2009]. CSCs have been reported to be highly resistant to certain antitumor therapies such as chemotherapeutic agents and IR [Gupta *et al.*, 2009]. Biomarkers for HCC CSCs have recently been identified and may offer insight into how CSCs are resistant to certain therapies and then contribute to recurrent tumors. A recent study concluded that an EpCAM<sup>+</sup> (epithelial cell adhesion molecule) AFP<sup>+</sup> (alpha-fetoprotein) HCC subtype had features of hepatic stem/progenitor cells. Further, they showed that EpCAM<sup>+</sup> cells were able to self-renew, differentiate, and were capable of initiating invasive HCC in *in vivo* studies [Yamashita *et al.*, 2009]. Interestingly, NdrG1 knockdown cells show decreased EpCAM expression compared to control cells in a HCC cell line derived from a portal vein tumor thrombus (PVTT) (Figure A4).



**Figure A. NdrG1 involvement in HCC tumorigenesis**

(1) MTS assay of PVTT-1 cells with NdrG1 shRNA (2) Western blot of cell lines used in MTS assay (3) Upregulation of NdrG1 in PVTT-1 cells after 10 Gy ionizing radiation (4) Downregulation of EpCAM in PVTT-1 cells with NdrG1 shRNA.

We hypothesize that NdrG1 may interact with EpCAM and contribute to EpCAM<sup>+</sup> HCC cell tumorigenic phenotypes. While NdrG1 has been shown to be mostly cytoplasmic in a variety of different cell types, IHC of NdrG1 in HCC tissue shows distinctive membrane staining, supporting the idea that NdrG1 may interact with the membrane-bound EpCAM in cancer cells [Akiba *et al.*, 2008]. We are interested in further investigating a possible connection between EpCAM and NdrG1 in putative HCC CSCs in order to understand the role of NdrG1 in HCC. Future experiments include identifying possible interactions between NdrG1 and EpCAM and whether these interactions are direct or indirect through immunoprecipitation

assays. We also want to further identify the role of EpCAM in HCC by knocking it down through shRNA and observe the effects of HCC cell viability. Results from these experiments may offer more insight into the role of NdrG1 in HCC tumorigenesis and recurrence.

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## **Biography**

Valerie Forbes Curtis was born to Daphne Forbes and Edward Curtis on March 10, 1984 in New Orleans, Louisiana. In 1995, her family moved to Raleigh, North Carolina where Valerie graduated from high school in 2002. Later that year, she attended Boston University in Boston, Massachusetts. On May 14, 2006, she graduated from Boston University Magna Cum Laude with a Bachelor of Arts in Biochemistry and Molecular Biology and a minor in Psychology. Valerie has recently submitted and is currently revising a manuscript for PLoS ONE entitled “A PK2/Bv8 Antagonist Suppresses Tumorigenic Processes by Inhibiting Angiogenesis in Glioma and Blocking Myeloid Cell Infiltration in Pancreatic Cancer”.